MATING STRATEGIES OF FEMALE CETACEANS

A Dissertation

by

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ABSTRACT

This dissertation provides broad insights on aspects of sexual selection in cetaceans (whales, dolphins, and porpoises) and addresses the gap of knowledge regarding female mating strategies. A comparative approach is applied to investigate the coevolution of mating strategies between the sexes and between anatomy and behavior, using dusky dolphins (*Lagenorhynchus obscurus*) as a model species. There are several key outcomes: (1) A heuristic framework is developed for the coevolution of mating strategies, in which males have low monopolization potentials of females, females evolved evasive behavioral maneuvers, males evolved large relative testes sizes, and females evolved convoluted vaginas. (2) Female mating behaviors are assessed in the context of exploitative scramble competition. Female dusky dolphins display evasive behavioral maneuvers during mating chases and discriminate among male behaviors. (3) A standardized measurement protocol is developed for female reproductive tracts and the microstructure of the unusual vaginal folds found in cetaceans is explored. The vaginal morphology of common bottlenose dolphins (*Tursiops truncatus*) is conserved across sexual maturity and reproductive states and consists of one caudally-oriented vaginal fold. Vaginal fold tissue is comprised of smooth (autonomic origin), not skeletal muscle (somatic origin). (4) Issues of scaling are examined while controlling for phylogenetic relatedness across 19 species. Vaginal lengths and vaginal fold lengths are correlated with body length but not each other, setting the stage to test functional hypotheses. (5) Reproductive anatomy (post-copulatory mechanism) and mating

behavioral effort (pre-copulatory mechanism) are explored across dusky dolphins, bottlenose dolphins, and harbor porpoises (*Phocoena phocoena*). A pattern appears between vaginal complexity and testes size. However, female pre-copulatory traits (behavioral repertoire size and intensity) do not match the trends predicted based on post-copulatory traits. Female dusky dolphins display the highest behavioral effort. Behavioral variation across species may reflect different environmental conditions and indicate that females, like males, may use several pre- and post-copulatory mechanisms to control paternity. (6) Overall, dusky dolphins adhere to the proposed heuristic framework. This dissertation demonstrates that female genitalia can provide important insights into cetacean mating strategies, and emphasizes the value of integrative approaches that examine coevolutionary interactions between the sexes and between anatomy and behavior.

DEDICATION

To my father, who initiated my love of boating and marine life, inspired my work ethic, was my moral compass, and taught me to appreciate the marvels of life. I miss you every day.

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CHAPTER I

INTRODUCTION

Summary

The broad variation in mating strategies of animals is the product of coevolution between male and female traits (e.g., Wiley and Poston, 1996; insects, Arnqvist and Rowe, 2002a; fish, Houde and Endler, 1990; waterfowl, Brennan et al., 2007; primates, Nunn, 1999; ungulates, Bro-Jørgensen, 2011). Strategies are genetically-based decision rules, while tactics are phenotypic patterns that can be manifest as behavioral, morphological, or physiological traits (Davies et al., 2012). Models of the diversity of mating strategies should include both sexes, as male and female strategies can be strongly inter-dependent (Bro-Jørgensen, 2011). Observations of behavioral or morphological changes in one sex that are correlated with changes in the other sex have opened a window to explore underlying processes of evolution. Application of the comparative approach across populations and species can enhance understanding evolutionary processes by highlighting environmental interactions with the genotype.

Although some existing conceptual frameworks of the evolution of mating strategies in cetaceans (whales, dolphins, and porpoises) may incorporate physical (e.g., ecological) and socio-sexual competition (e.g., coercive mating) environments, they do not address the coevolution of anatomical and behavioral traits between the sexes (e.g., Magnusson and Kasuya, 1997; Connor et al., 2000a; Whitehead and Mann, 2000;

Gowans et al., 2007). It is generally hypothesized that only female cetaceans offer parental care (Connor et al., 2000a) and that mating systems are comprised of primarily polygynous (one male mates with multiple females) or polygynandrous (males and females mate with multiple partners) relationships (Wells et al., 1999). Despite the logistical challenges of direct observations (e.g., Lanyon and Burgess, 2014; Orbach et al., 2014, 2015a), males are known to use a variety of non-mutually exclusive pre- and post-copulatory mating tactics to secure paternity (Connor et al., 2000a; Dines et al., 2015; Table 1-1). The mating tactics of female cetaceans remain relatively unknown (Connor et al., 2000a; Boness et al., 2002; Schaeff, 2007; Mesnick and Ralls, 2009), although some diverse female mating tactics have been hypothesized (Table 1-2).

Overview of Chapters and Research Objectives

The goal of this dissertation is to increase our knowledge of female mating tactics among cetaceans. I develop a heuristic framework with which to examine the coevolutionary nature of female and male mating strategies that include behavioral and anatomical traits. Dusky dolphins (*Lagenorhynchus obscurus*) are used as a model species and a comparative approach is applied to assess variation in tactics arising from different ecological and socio-sexual environments.

Table 1-1. General male mating tactics of cetaceans for intrasexual competition.

Male Mating Tactic	Tactic Definition	Examples/Possible Evidence	Species	Reference
Display Competition	Males engage in courtship displays and compete for the attention of females using morphological or behavior signals that are assumed to reflect genetic quality, dominance, readiness to breed, or access to resources	Sexual dimorphism in size and shape of postanal humps, dorsal fins, and caudal peduncles	Dall's porpoise (*Phocoenoides dalli)*	Jefferson, 1990
		"Slow clicks"	Sperm whale (*Physeter macrocephalus*)	Weilgart and Whitehead, 1988; Whitehead, 1993
		Songs on breeding grounds	Humpback whale (*Megaptera novaeangliae*)	Clapham, 1996; Darling and Bérubé, 2001
		Stick carrying	Amazon river dolphin (*Inia geoffrensis*)	Martin et al., 2008
Contest Competition	One or more males limit the access of other males to reproductive females through fights or aggressive behaviors	Observations of violent intrasexual interactions	Common bottlenose dolphin (*Tursiops truncatus*)	Parsons et al., 2003
		Tooth rake marks	Odontocetes	MacLeod, 1998
		Weaponry- "battle teeth"	Some beaked whale (Family Ziphiidae)	McCann, 1974; Heyning, 1984; Pitman, 2008
		Weaponry- callosities	North Atlantic right whale (*Eubalaena glacialis*)	Kraus and Hatch, 2001
			Southern right whale (*E. australis*)	Payne and Dorsey, 1983
		Weaponry- tusks	Narwhal (*Monodon monoceros*)	Silverman and Dunbar, 1980; Gerson and Hickie, 1985

Table 1-1, Continued.

Male Mating Tactic	Tactic Definition	Examples/Possible Evidence	Species	Reference
Endurance Competition	Males attempt to outlast their rivals for the duration of a "war of attrition"	Mate guarding/ Consortships	Dall's porpoise (*P. dalli*)	Willis and Dill, 2007
			Indo-Pacific bottlenose dolphin (*T. aduncus*)	Connor et al., 1996
Scramble Competition	Competition to disperse and find sexually receptive females and mate with as many as possible within typically brief time constraints	Maneuverability during mating chases	Dusky dolphin (*Lagenorhynchus obscurus*)	Markowitz et al., 2010
		Surface active groups	Humpback whale (*M. novaeangliae*)	Clapham et al., 1992
			North Atlantic right whale (*E. glacialis*)	Kraus and Hatch, 2001
Sperm Competition	A post-copulatory tactic that occurs inside the female reproductive tract. Males with higher quality or higher quantities of sperm that can displace or dilute their rivals' sperm succeed in fertilizing the most ova	Strong seasonal testes mass and cellular activity patterns	Common dolphin (*Delphinus delphis*)	Murphy et al., 2005
			Gray whale (*Eschrichtius robustus*)	Rice and Wolman, 1971
Alternative Reproductive Strategies	Males with poor competitive abilities may sneak copulations or intercept females as "satellite" males that remain close to males engaged in courtship displays	No evidence in cetaceans	[1]	

[1] See chapter V for male "interceptions" of female harbor porpoises (*Phocoena phocoena*)

Table 1-2. Hypothesized general female mating tactics of cetaceans for intersexual selection.

Female Mating Tactic	Tactic Definition	Examples/Evidence	Species	Reference
Signal Discrimination	Females evaluate and select among prospective mates based on a male trait (e.g., phenotypic "signals", dominance, access to resources)	Extended mating chases led by females that may be used to evaluate male maneuverability	Dusky dolphin (*Lagenorhynchus obscurus*)	Markowitz et al., 2010; Orbach et al., 2014
		Songs on breeding grounds	Humpback whale (*Megaptera novaeangliae*)	Tyack, 1981; Chu, 1988; Clark and Clapham, 2004
Mate Choice Copying	Females increase or decrease their likelihood of mating with a particular male based on observing the mating behavior of other females	Suggested by patterns of paternal relatedness within matrilineal groups	Sperm whale (*Physeter macrocephalus*)	Richard et al., 1996
Evasive Behaviors	Females actively avoid copulation with males through body positioning or use of their habitat	Females fled from pursuant males, moved to shallow waters where males could not fit beneath them, rolled ventrum-up, and raised flukes in the air so their genital groove was inaccessible	Dusky dolphin (*L. obscurus*)	Orbach et al., 2015a/ chapter II
			Humpback whale (*M. novaeangliae*)	Mattila et al., 1988
			North Atlantic right whale (*Eubalaena glacialis*)	Kraus and Hatch, 2001
			Southern right whale (*E. australis*)	Payne, 1995
Polyestry	Females have repeated or shifted estrous cycles during which they can conceive	Hypothesized physiological mechanism to obscure paternity, reduce sexual harassment costs, and deter non-parental infanticide	Indo-Pacific bottlenose dolphin (*Tursiops aduncus*)	Connor et al., 1996
Modified Genitalia	A post-copulatory tactic that occurs in the female reproductive tract	Complex vaginal folds potentially capable of moving semen	Cetaceans	Orbach et al., 2016/ chapter III, chapters IV and V

Chapter I provides an overview of the dissertation and lays out the heuristic framework with which to examine the coevolution of female and male mating strategies in dusky dolphins.

Chapter II determines if female dusky dolphins display evasive behavioral maneuvers in the context of male exploitative scramble competition. My objectives were to: 1) ascertain if females have the potential to exercise active mate choice, and 2) evaluate if mate choice can be demonstrated through maneuverability. I collected and analyzed video recordings of mating group interactions and assessed the frequencies and types of behaviors (non-evasive vs. evasive), differences in the transition probabilities of behaviors leading to copulation, and variation in female responses to male behaviors.

Chapter III investigates vaginal morphology patterns in the dissected reproductive tracts of common bottlenose dolphins (*T. truncatus*). The common bottlenose dolphin was used instead of dusky dolphins because of access to larger sample sizes. My objectives were to: 1) develop a standardized measurement protocol for the reproductive tracts of female cetaceans, 2) examine morphological patterns of variation across sexual maturity state, reproductive state, and geographic area of stranding, 3) assess vaginal microstructure to determine if vaginal folds are under somatic muscular control and capable of selective sperm movement, and 4) compare dissection and computed tomography (CT) scan methods.

Chapter IV assesses if vaginal folds are stereotyped across the cetacean phylogeny. It is necessary to establish if vaginal morphology scales with body size before addressing hypotheses about the relative importance of the physical and socio-

sexual competition environments in shaping vaginal fold variation. My objectives were to: 1) evaluate scaling relationships between vaginal folds, vaginal lengths, and total body lengths while controlling for phylogenetic effects, and 2) investigate variation across species.

Chapter V combines measurements of mating behavior with reproductive anatomy and explores their association and possible trade-offs across three species. My objectives were to: 1) examine species-specific differences in female and male post-copulatory traits (vaginal complexity/testes size), 2) predict variation in female pre-copulatory traits (behavioral effort to evade males) based on post-copulatory traits, and 3) review species-specific differences in female pre-copulatory traits using video recordings of mating events.

Chapter VI synthesizes the previous chapters and revisits the applicability of the heuristic framework proposed for the coevolution of mating strategies in dusky dolphins. The importance of the research is reviewed and future behavioral and anatomical research directions with the potential to broadly advance the field of sexual selection are indicated.

Heuristic Framework

I propose a heuristic framework with which to examine the coevolution of female and male mating strategies in dusky dolphins. The framework incorporates pre-copulatory (behavioral) and post-copulatory (morphological) traits of both sexes. When males can monopolize access to multiple females, the allocation of fixed energy budgets towards

traits that enhance acquisition of mates are often traded-off against traits that enhance fertilization success (e.g., Parker et al., 2013). This pattern was found in cetaceans (Dines et al., 2015). It is unclear if female cetaceans also experience such trade-offs in pre- and post-copulatory traits. The proposed heuristic framework has four components- males have low monopolization potential of females, females evolved evasive behavioral maneuvers, males evolved large relative testes sizes, and females evolved convoluted vaginas. Chapters II through V provide supporting evidence for the framework, and chapter VI revisits and synthesizes the data.

Males Have Low Monopolization Potential of Females

Male dusky dolphins in the waters off Kaikoura, New Zealand, engage in exploitative scramble competition, during which they maneuver for a proximate position next to an (presumed) ovulating female and rarely engage in aggressive interactions (Markowitz et al., 2010; Orbach et al., 2014, 2015a, 2015b). As mating group size increases above an optimum of 6 males, intrasexual competition increases, and each male's potential to exclude other males from copulating with the female declines (Orbach et al., 2014, 2015c). However, if the group size gets too small, males extend more energy to keep pace with the female (Orbach et al., 2014). The low monopolization potential of female dusky dolphins off Kaikoura is largely driven by the distribution of spatially and temporally reliable prey patches that do not require resource defense (Dahood and Benoit-Bird, 2010) and low predation pressure due to large aggregations of dolphins (Srinivasan and Markowitz, 2010). Additionally, females are able to use their 3-

dimensional environment and the lack of physical barriers in the water column to evade males by diving.

Females Evolved Evasive Behavioral Maneuvers

The low potential of males to monopolize female dusky dolphins in the waters off Kaikoura could enable females to evade undesirable prospective mates without consequences of received physical aggression or other direct costs (e.g., Watson, 2005). Female dusky dolphins have less conspecific-acquired markings than males (Orbach et al., 2015b) and aggressive male behaviors directed towards females in mating groups have not been observed off Kaikoura (Markowitz et al., 2010; Orbach et al., 2015a, 2015b). Accordingly, if females are autonomous and their movements are not controlled by males, they are predicted to exhibit high frequencies of evasive behavioral maneuvers and variable responses to male mating behaviors that reflect active mate choice and control of paternity (chapter II).

Males Evolved Large Relative Testes Sizes

Males evolved large testes sizes that provide a mechanism to compete for paternity inside the female's reproductive tract. Large relative testes sizes are a strong indicator of male investment in sperm competition and multi-mate breeding systems across many taxonomic groups (Birkhead and Møller, 1998; birds, Møller, 1991; fish, Stockley et al., 1997; Pitcher et al., 2005; primates, Hartcourt et al., 1995; bats, Hosken, 1997), although not the only indicator (e.g., Pintus et al., 2015). Odontocetes (toothed whales) possess

large relative testes sizes compared to terrestrial mammals, with species located above the isometric line predicted by body size for mammals (Kenagy and Trombulak, 1986; Aguilar and Monzon, 1992). There is a large range of relative testes sizes among cetaceans (Brownell and Ralls, 1986; Aguilar and Monzon, 1992; Connor et al., 2000a; Dines et al., 2015). Breeding dusky dolphins have among the highest reported testes-to-body size ratios among mammals, at 5.4 - 8.5% of their body mass (Cipriano, 1992; van Waerebeek and Read, 1994). This suggests that sperm competition is a particularly important male mating tactic for dusky dolphins (Markowitz et al., 2010).

Females Evolved Convoluted Vaginas

Female cetaceans evolved complex vaginal morphologies that may function as physical obstacles to sperm movement (Clarke et al., 1994). Cetaceans possess unusual vaginal folds of varying relative sizes that are not found in other marine mammal lineages (chapter IV; appendix A). Modified genitalia can be a strong indicator of a coevolutionary "arms race" between the sexes (Eberhard, 1996; Hosken and Stockley, 2004). For example, the genital elaborations (clockwise spirals) in female waterfowl function as a physical barrier to phallus penetration during forced copulations (Brennan et al., 2007, 2010). The forms of cetacean vaginal folds may yield insights on mechanisms of paternity control (chapters III and IV).

This proposed heuristic framework is consistent with models of the coevolution of mating strategies developed for other taxonomic groups that have not yet been systematically applied to cetaceans. Whether the framework's components arose in a linear sequence or divergent branches during evolutionary history remains to be examined. Identification and validation of components of the framework are a critical initial step to resolve evolutionary history questions as additional data become available for more species.

CHAPTER II

EVASIVE BEHAVIORS OF FEMALE DUSKY DOLPHINS (*LAGENORHYNCHUS OBSCURUS*) DURING EXPLOITATIVE SCRAMBLE COMPETITION*

Summary

When males engage in scramble competition, are females non-evasive recipients of male coercion or evasive? Small groups of male dusky dolphins (*Lagenorhynchus obscurus*) engage in exploitative (non-interference) scramble competition for access to estrous females near Kaikoura, New Zealand. We conducted behavioral sequence analyses of videos of 48 mating groups with continuous records and focal individual follows of females. We determined (1) the frequency and type of behaviors (non-evasive vs. evasive), (2) the variation in simple transition probabilities of behaviors leading to a copulatory position associated with female resistance and non-resistance, and (3) the variation in female responses to male behaviors throughout focal follows. Females exhibited frequent active evasive manoeuvers following male behaviors. Copulation sequences were highly variable and most complex when terminated by females. Females altered responses to male signals, one aspect of mate choice potentially favoring male endurance.

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*Reprinted with permission from "Evasive behaviours of female dusky dolphins (*Lagenorhynchus obscurus*) during exploitative scramble competition" by Orbach DN, Packard JM, Kirchner T, and Würsig B, 2015. Behaviour, 152, 1953-1977, doi: 10.1163/1568539X-00003310, Copyright [2015] by Brill. The text of the published article has been amended to U.S. spelling.

Introduction

Female control of paternity has received considerably less attention than male control (reviewed in Jennions and Petrie, 1997; Clutton-Brock and McAuliffe, 2009; Bro-Jørgensen, 2011). Among polygamous mammals, mechanisms of female control of paternity include, but are not limited to, responses that reduce the risk of harassment (Clutton-Brock and Parker, 1995), active choice of displaying males (Clutton-Brock and Parker, 1995), post-copulatory sperm selection (Eberhard, 1996), and evasive tactics (Chilvers et al., 2005). In polygynous (one male mates with several females) and polygynandrous (multiple males mate with multiple females) mammals, sexual coercion and intrasexual male competition may mask a female's mating preferences (Clutton-Brock and McAuliffe, 2009).

Mating tactics are largely unknown for most cetaceans (reviewed in Boness et al., 2002; reviewed in Schaeff, 2007; reviewed in Mesnick and Ralls, 2009). The few existing studies of behavioral mechanisms of paternity control in cetaceans have focused mostly on overt male contest competition (reviewed in Schaeff, 2007) and/or on bottlenose dolphins (*Tursiops* sp.; reviewed in Connor et al., 2000b). Male Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) in Shark Bay, Western Australia, form small stable first-order alliances and cooperate to sequester sexually mature females and monopolize access to them (Connor et al., 1992a, 1992b, 2000b; Connor and Krützen, 2015). Females that resist males can incur physical violence and are coerced to remain with alliance members by threat displays from males (Connor and Smolker, 1996; Connor et al., 1996). Female tactics to control paternity are subtle and may include

polyestrous cycling (Connor et al., 1996). Females appear to be constrained in their mate choice (Connor et al., 2000b), and fit a 'non-evasive female' model, consistent with other mammalian groups in which males monopolize females with aggression or weaponry (e.g., primates; Muller and Wrangham, 2009; see Mesnick, 1997 as exception). There is a deficit of systematic studies that assess female active (behavioral) mate choice among cetaceans (reviewed in Whitehead and Mann, 2000; reviewed in Schaeff, 2007).

During scramble competition, males jostle for a proximate position near a fertile female, followed by a series of actions directed toward the female. The costs incurred by females due to coercive males (e.g., sequestering, forced copulations, harassment, punishment, and intimidation; Clutton-Brock and Parker, 1995) may be low in non-contest contexts like scramble competition, and females may overtly discriminate between prospective mates. Female southern right whales (*Eubalaena australis*) and humpback whales (*Megaptera novaeangliae*) fled from males and led them on exhausting high-speed mating chases (Tyack and Whitehead, 1982; Payne, 1995). Females also moved to shallow waters where males could not fit beneath them, rolled to turn belly-up at the surface of the water, or lifted their tail out of the water so their genital grooves were inaccessible to males (Darling, 1983; Mattila et al., 1988; Payne, 1995). These female maneuvers were evasive because they physically blocked access of the penis to the genital opening. Female maneuvers during scramble competition are not widely documented among mammals and it is not clear if females simply evade all

pursuing males or subtly manipulate the likelihood that some males are more likely to gain access to them than others.

Sexually-monomorphic dusky dolphins (*Lagenorhynchus obscurus*) near Kaikoura, New Zealand, provide an opportunity to evaluate the potential extent of active female mate choice in the context of male exploitative competition. During exploitative scramble competition, a male removes the female from the resource pool by mating with her during ovulation. Unlike conventional interference scramble competition models, male dusky dolphins do not appear to intimidate their rivals (Markowitz et al., 2010; Orbach et al., 2014). Interference events among males are uncommon during mating chases, although male dusky dolphins receive more overt aggression than females (Orbach et al., 2015b). Dusky dolphins mate in ventral contact with the female ventrum-down at or near the surface of the water (Markowitz, 2004). Mating groups range in size up to fifteen dolphins but typically consist of four adult males chasing one sexually mature female for a proximate position underneath her (Orbach et al., 2014). Females only mate with one third of the males in mating groups (Markowitz, 2004). The copulation rate per male declines with the number of males in a group (Orbach et al., 2015c). Copulations are brief (mean = 4.9 sec) while mating chases are considerably longer (mean = 15.8 min) and consist of multiple copulation events (Orbach et al., 2014).

We hypothesize that female dusky dolphins evade male coercion. We assess if females meet the predictions of 'non-evasive female' or 'evasive female' models. Characteristics of the 'non-evasive female' model would be (1) behavioral repertoires

with more non-evasive than evasive behavior types, (2) more frequent occurrences of non-evasive than evasive behavior types, and (3) random responses to male signals. If dusky dolphin mating behavior better fits the 'evasive female' model, we wanted to determine how females were evasive in (1) copulatory sequences with and without resistance and (2) overall responses to male behaviors.

Material and Methods

Study Population

Dusky dolphins inhabit the open-ocean embayment around the Kaikoura Peninsula on the northeast coast of the South Island of New Zealand (42°25"S 173°41"E). They aggregate in large groups (over 1,000 dolphins) with fission-fusion social structures (Würsig et al., 2007). There is a spatio-temporal separation of behavioral states. The dolphins feed offshore at night on the mesopelagic myctophids and squids of the deep scattering layer that rises from the depths of the Kaikoura Canyon towards the surface of the water (Benoit-Bird et al., 2004, 2009). Observations of daytime foraging are rare (Markowitz, 2004, 2012). Daytime activities are predominantly socializing and resting in shallow inshore waters (Markowitz, 2004).

Data Collection

Dusky dolphin mating groups were followed near Kaikoura from October 2013 through January 2014, coinciding with the peak of mating behavior during the austral summer (Markowitz, 2004). Mating groups can be distinguished from small (< 50 individuals)

non-mating groups by observations of re-orientation leaps (see Table 2-1), the presence of males swimming inverted with their penises everted, and attempted copulations (Markowitz, 2004; Orbach et al., 2014). Satellite mating groups form near the main pod of dolphins. Juveniles are occasionally, and calves are rarely, observed in mating groups (Markowitz, 2004). Focal follows were conducted on mating groups that maintained a constant group size and composition and a maximum spacing of 10 m between dolphins (Smolker et al., 1992). Dolphins were approached from a 6-m rigid-hull inflatable vessel with an 80-hp 4-stroke outboard engine. We travelled parallel to the groups to reduce behavioral disturbance (Markowitz et al., 2009). Follows of mating groups were conducted between 8:00-11:30 and 13:30-17:00 in good boating conditions (<3 Beaufort) when the dolphins could be tracked continuously. There is a mandated, mid-day, 2-h no-approach time due to the prevalence of resting behavior in the dolphin population (Lundquist et al., 2012), so no data were gathered between 11:30 and 13:30. Water visibility was typically 5-8 m but could range from 2-15 m. Follows were terminated when the group size changed, mating behaviors ceased, or boating conditions became unsafe. Strict adherence to the operating rules in the New Zealand Marine Mammals Protection Act 1978, Marine Mammals Protection Regulations 1992, and local dolphin conservation guidelines was maintained at all times (Childerhouse and Baxter, 2010). No permit was required for our observational study.

Table 2-1. Repertoire of behaviors observed in mating sequences of dusky dolphins, sorted by frequency within each category (female, male, synchronized group).

Category	Behavior Type	No. of Events	Definition
Female	Tail slap†^	453	Female raises her tail out of the water and strikes it against the surface of the water with force, creating a noisy splash.
	Direction change†*	179	Female quickly moves non-linearly through the water, abruptly switching direction one or more times.
	Body roll	150	Female rotates her body along her longitudinal axis (e.g., rolls onto her back).
	Re-orientation leap†^*	54	Female leaps vertically out of the water and re-enters head-first nearby. Her ventral surface is oriented down. Her whole body clears the surface of the water and no loud splash is generated.
	Speed burst†^*	15	Female moves horizontally and at high speed at the surface of the water with minimal changes in direction.
	Spy hop†^*	12	Female is positioned vertically in the water with her eyes and rostrum above the surface.
Male	Inverted swim†^*	1143	Male swims in a ventrum-up body position (not under a dyad in copulatory position, see below).
	Push female to surface†*	586	Inverted male pushes female up vertically while swimming ventrum-to-ventrum with her such that her dorsal region is above the surface of the water.

Table 2-1, Continued.

Category	Behavior Type	No. of Events	Definition
	Swim under pair	59	Male swims inverted below and within one body-width of a dyad in a copulatory position.
	Swim under leaping female	33	Male swims inverted below a leaping female.
	Interference*	31	Male moves in a manner resulting in separation of a dyad in copulatory position (e.g., blocking the direction of swimming, rolling over the dyad, etc.).
Synchronized group	Copulatory position[#]	664	Two dolphins press bellies together in ventral contact with their genitals aligned. The penis may or may not penetrate the vagina.
	Coordinated re-orientation leap*	246	Two or more dolphins do a synchronous vertical leap out of the water (see female re-orientation leap).
	Group dive*	91	All dolphins dive and/or are out of view below the surface for at least 30 consecutive seconds.
	Tight circles	17	Two or more dolphins swim in a circle oriented in the same direction (usually the radius is 1-2 body lengths).

† Defined in Markowitz (2004)
^ Defined in Markowitz (2012)
* Defined in Markowitz et al. (2010)
[#] Defined in Markowitz (2004) and Markowitz et al. (2010) as ventral presentation

Mating groups were spotted by three researchers scanning the horizon while travelling parallel to the shoreline. Groups were approached within approx. 5 m and the corresponding time, GPS coordinates, group size, and group composition were documented. A Garmin GPSMAP 76 GPS was used to record the time and geographic coordinates. When the group size changed, a new follow commenced. Mating groups were observed when the water visibility was sufficiently clear that we could confirm no fission or fusion of other dolphins onto the group. Dolphins were classified *in situ* as adults, juveniles (> one-third adult body length and ≤ two-thirds adult body length), or calves (≤ one-third adult body length; Würsig and Würsig, 1980). The sexes were distinguished based on viewing their anal-genital slits or genitalia and body positioning during copulation events (males ventrum-up and females ventrum down; Markowitz et al., 2010; Orbach et al., 2014). The sexes could not be confirmed by genetic sampling because biopsy techniques elicit short-term behavioral responses that can disrupt mating behaviors (Krützen et al., 2002).

It is unlikely that focal females in a group were misidentified. While it is conceivable that some males were positioned in the ventrum-down 'female' body position during mating chases and copulations, female mimicry is rare and obvious. On one occasion, we observed a penis on the individual in the female body orientation. The male mimicking the female and the rest of the dolphin group behaved different than typical mating groups. Most dolphins in the group did not pursue the 'pseudo-female', the pursuing males were comparatively docile in their chasing efforts, and the 'pseudo-

female' demonstrated limited evasive behaviors. The video record of this group was not included in the analysis.

A Sony Handycam HDR-XR550V was used to record continuous videos of mating behaviors while travelling parallel to each dolphin group. We focused on following the female in the group and recording all her behaviors. Her individually-distinctive dorsal fin was visible the majority of the time, unlike males who were often inverted. The video camera was mounted on a shoulder pod to reduce camera shake. Detailed *ad libitum* narrations were recorded simultaneously with the videos including the observed behaviors, their initiation and termination, and the sex of the dolphin performing the behavior.

We acknowledge several constraints related to recording the complete sequences of mating behaviors necessary to meet the strict criteria of stationarity (Blumstein and Daniel, 2007). It was challenging to determine the identity of males when their individually-distinctive dorsal fins were not visible when they were inverted. Males did not have their penises exposed for the duration of the follow. Genitals were difficult to detect when individuals were ventrum-down at the water surface. We assumed all individuals in the ventrum-up position were males, even if the genitals were obscured. Subtle behavioral changes were obscured when males were ventrum-up beneath a female, at a distance, or due to water distortion. There were several males in each mating group, and it was not possible to track all their behaviors simultaneously. The issue of stationarity was addressed by treating data as incomplete sequences. We focused only on the co-occurrence of adjacent events in sequences, framing the question in terms of

"given that X occurred, what is the likelihood it was preceded by Y". The general female responses to male signals were used as precursors to mate choice because actual mate choice could not be determined under the field constraints of unknown individual identities.

Analysis

Forty-eight videos, each corresponding to a single focal follow of a mating group, were viewed and transcribed using the software Transana (version 2.51). We recorded metadata for each video, including group size, composition, and percent surface time. The percent surface time was calculated by subtracting the durations of all group dive intervals (from the moment the first dolphin dove to depth until any group member first resurfaced) from the total duration of the video (Laake et al., 1997). The video playback was slowed down by as much as 10x the original speed and time stamps were added to the transcripts.

The list of behavior types was developed using previously defined behaviors (Markowitz, 2004, 2012; Markowitz et al., 2010) and was modified based on personal observations during previous field seasons (Table 2-1). For example, we could not reliably distinguish between copulatory events with or without intromission (as defined by Markowitz, 2004), so used the more conservative definition of 'copulatory position'. Behaviors were categorized as female-specific, male-specific, or synchronized in both females and males (Table 2-1). The behavioral repertoire used for this analysis included 6 female behavior types, 5 male behavior types, and 4 synchronized group behavior

types (Table 2-1). It is a subset of the complete behavior repertoire for dolphins, which includes affiliative behaviors (e.g., flipper touching), which were not observed in the videos we analyzed.

All occurrences of the defined behaviors were transcribed as a continuous sequence using the video for each mating group. In a few cases, behaviors narrated by the observer were included in the transcripts when visibility was reduced by the challenges associated with filming in the field (e.g., moving platform, moving animals, small field of view). Repeated bouts of a single behavior type were recorded as separate events with the exception of speed bursts (Table 2-1). We noted missing information associated with each behavioral event as 'unknown', such as the identity of individuals performing a re-orientation leap at a distance. If there was a gap of more than 3 s before or after events (e.g., the dolphins in the video did not exhibit any defined behaviors), we recorded the behavior as 'unknown'. Only behavior types that were observed at least twice during the study period were included in Table 2-1 and the subsequent analyses. For example, males were observed herding a female against the shore on only one occasion, so the behavior was not included.

To examine female responses to male signals, we used an informatic approach consistent with established procedures for sequence analysis (Bakeman and Gottman, 1986; Lehner, 1996; Blumstein and Daniels, 2007). In this conceptual framework, the basic questions are whether behavior types are equiprobable and whether a target behavior type is equally likely to be preceded or followed by any other behavior type. The statistical models are robust to the relaxation of assumptions of stationarity. We

assumed no variation associated with individuals and mating groups in the likelihood of events occurring in behavioral sequences. We did not examine the effects of external variables on transition probabilities. Accordingly, our study design in not subject to concerns of pseudo-replication or inadequate controls.

Frequency of Behaviors

We tested the hypothesis that the frequencies of behavior types were equiprobable within each category in Table 2-1 (i.e., female, male, and synchronized groups). This hypothesis corresponds to the maximum zero-order uncertainty in a Markovian analysis (Blumstein and Daniel, 2007). The number of occurrences of all behavior types was tallied for each video and pooled across all videos. A chi-square goodness of fit test was calculated for each of the three categories in Microsoft Excel (2010), based on the equation in Table 14.14 of Lehner (1996):

$$\sum \frac{(O-E)^2}{E},$$

where O is the observed value and E (total number of behavioral events divided by the number of behavior types) is the expected value.

We examined the resulting repertoire of behaviors and scored behavior types as relatively frequent or rare and as evasive or non-evasive. Our criterion for a rare behavior type was a total tally of less than 2% of all events recorded. We define 'evasive' as any behavior that interrupts a sequence of events that culminate in copulation. In contrast, non-evasive behaviors facilitate copulation through decreased

distances and increased body contact. This distinction is similar to the concepts of receptive and proceptive behavior in female mammals (Beach, 1976). Evasive behaviors indicate low receptivity. Non-evasive behaviors indicate high proceptivity. Since females were also part of synchronized groups, we used the same procedure to score behavior types within the synchronized group category.

Sequences of Behaviors Prior to Copulation

We tested the hypothesis that any behavior type was equally likely to follow any other behavior type. This hypothesis corresponds to first-order uncertainty in Markovian processes, and examines the serial dependence between adjacent behavior types (Blumstein and Daniel, 2007). We reasoned that high first-order uncertainty (e.g., randomness) would fit the 'non-evasive female' more than the 'evasive female' model. We used overlapped sampling to tally the frequencies of two events occurring next to (i.e., immediately before or after) in a temporal sequence. Overlapped sampling refers to the process of sliding a two-event sampling window along the transcript of a sequence (e.g., A, B, C, D) and tallying the occurrence of subsequent pairs (e.g., AB, BC, CD) in a matrix (Lehner, 1996; Blumstein and Daniel, 2007).

We tested the prediction that adjacent events would occur at higher probabilities in sequences where females terminated copulations compared to 'normal' sequences where females did not terminate copulations. We coded each copulatory sequence as 'non-resistant' (i.e., terminated by the male or both copulating partners), 'resistant' (i.e., terminated by the female), or 'ambiguous'. We reasoned that copulatory bouts

terminated by the male or a mutual decision would indicate higher female receptivity than bouts terminated by the female. We did not specify the behavior types that terminated the copulatory position, only the consequence (e.g., females initiated termination of a copulatory position, regardless if they achieved the termination with a tail slap or a body roll). We used the term 'resistant' to refer to a copulatory sequence in contrast to the term 'evasive' to refer to an individual behavior type.

We identified all copulatory sequences in the videos. The end of each sequence was the copulatory position. We worked backwards to identify the beginning of the sequence, when the male moved within one body length of the female. Females were generally not observed performing any behaviors (other than surfacing to breathe and swimming) before males were in close proximity. With the exception of sequences with interference and/or swim under copulating pair, the behavior of only one male was transcribed per copulatory sequence. We excluded from further analysis the ambiguous sequences (N = 554), that did not meet our quality standards (e.g., far away, partially out of frame, etc.), or were terminated mutually in an active manner (mutual evasion) or by a third individual. Group size was recorded for each copulatory sequence.

Observed frequencies for transitions between adjacent events were tallied in separate matrices for resistant and non-resistant copulatory sequences. Simple probabilities determine the probability of an adjacent event occurring in a matrix. Simple probabilities were calculated by dividing the observed frequencies by the total number of adjacent events (Blumstein and Daniel, 2007). The matrices were constructed by starting at the end (copulatory position) and sliding the 2-event window backwards to add the

immediately preceding behavior types. This process was repeated for each behavior type. The matrices were presented visually as kinematic diagrams (Lehner, 1996; Blumstein and Daniel, 2007). Arrows were drawn between behavior types to indicate directionality. The widths of the arrows correspond to the values of simple probabilities. Simple probabilities below or above/equal to the threshold value of 0.1 (recommended by Blumstein and Daniel, 2007) were indicated by solid or dashed lines, respectively.

Female Responses to Male Signals

We tested the hypothesis that female responses were independent of preceding male behaviors in the dataset as a whole, including sequences that did not end in copulatory position. The 'non-evasive female' model would be supported by random associations of male and female behavior types. Nonrandom associations would indicate some level of decision-making based on information received from the male and support the 'evasive female' model. We analyzed signal/response contingencies by identifying adjacent events where female responses were preceded by male signals. The co-occurrences of all response behaviors (female and synchronized group) and the immediate preceding male mating behaviors were tallied.

A log-likelihood ratio test was used to determine whether the overall association of female responses and male signals differed from what would be expected by chance. Assumptions are that: (1) observations are dependent, (2) categorical variables are mutually exclusive, and (3) expected frequencies for each variable are above 5. We used

Microsoft Excel (2010) to calculate the G-square statistic using equation 4.2-2 in Bishop et al. (1975),

$$G^2 = 2\sum_i x_i \log \frac{x^i}{\hat{m}^i}$$

We examined all occurrences of five target female behavior types: tail slap, direction change, body roll, re-orientation leap, spy hop/speed burst (pooled together to meet expected frequency requirements), and copulatory position. We included copulatory position but not the other synchronous group behavior types because (1) copulation acceptance is a decision made by the female in response to one male, (2) the other synchronized group behaviors involved one or more males coordinating their behaviors with the female, and (3) copulation was the only non-evasive behavior type exhibited by females. We only included the two most recurrent male behavior types (inverted swim and push female to surface) in the log-likelihood ratio test because (1) inclusion of rare male behaviors violated the test assumption of expected frequencies above 5, (2) these two behavior types were directed at the female such that she was likely to receive information about the male, and (3) the other three male behavior types (swim under pair, swim under leaping female, interference) might not have been perceived by the female. To determine which adjacent events contributed to the significance of the log-likelihood ratio test, we calculated binomial z-scores as specified by Bakeman and Gottman (1986). Scores above 1.96 indicated adjacent events that occurred more frequently than expected by chance.

Results

Videos of 48 dusky dolphin mating groups yielded a total of 486 min of observations, with a mean surface time of 91% (SE = 2%). The modal group size was 4 adult males with 1 adult female and ranged from 2 to 25 dolphins. Mating groups were composed of all adults with the exception of one group that contained 5 adults and a single calf, and 4 groups that contained 1 juvenile (group sizes consisted of 3, 4, 4, and 7 adults). Recorded behavioral events (N = 3,733) were primarily in the category of male (50%) compared to female (23%) and synchronized groups (27%).

Frequency of Behaviors

Behaviors were not equiprobable for females ($GF\text{-}X^2_5 = 965.75$, $p < 0.001$), males ($GF\text{-}X^2_4 = 2617.16$, $p < 0.001$), or synchronized groups ($GF\text{-}X^2_3 = 958.86$, $p < 0.001$). The most frequent female behavior types (tail slap, direction change, and body roll) were evasive and had the potential to disrupt copulations (Fig. 2-1A). Even the rare female behavior types (re-orientation leap, speed burst, and spy hop) were not indicative of non-evasive receptive behavior. The most frequent male behavior types were inverted swim and push female to surface (Fig. 2-1B). One rare male behavior type was directed towards a female (swim under leaping female). The other rare male behavior types (swim under pair and interference) entailed maneuvers around a pair in the copulatory position. No direct conflict was observed within or between sexes. The frequent synchronous group behavior types included copulatory position, coordinated re-orientation leaps, and group dives (Fig. 2-1C). Synchronous tight circles were relatively

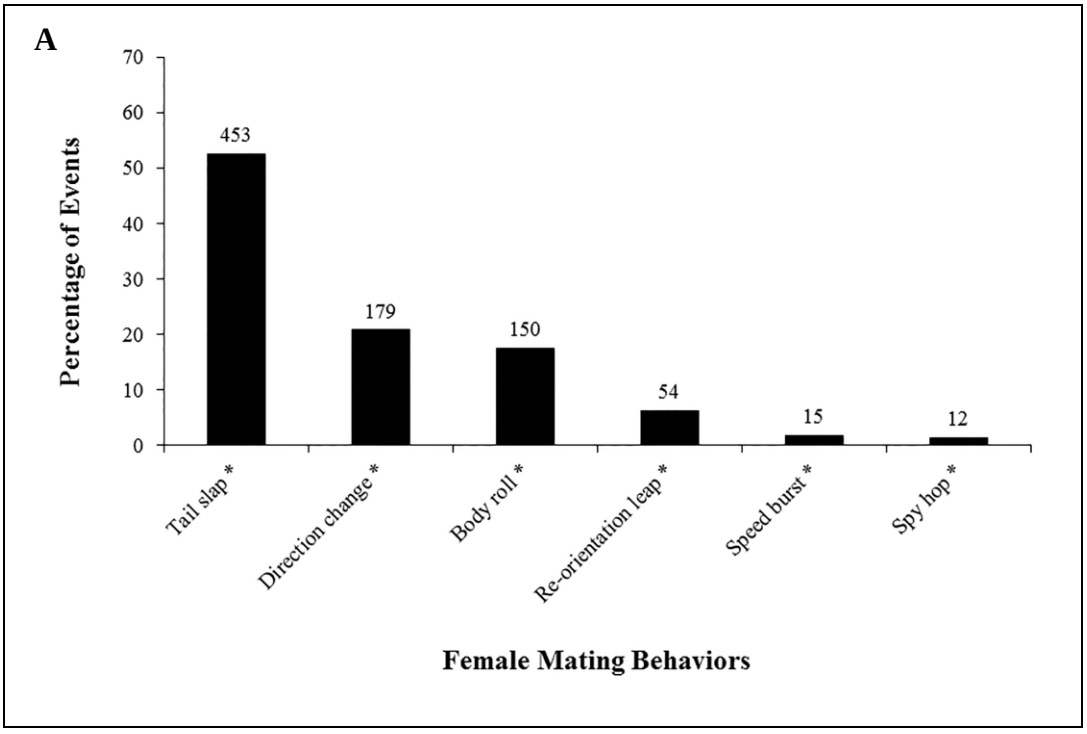

Figure 2-1. Frequency distributions of (A) female mating behaviors, (B) male mating behaviors, and (C) synchronized group mating behaviors. Frequencies are shown as percentages. The count of behavioral occurrences is listed above the bars for each behavior type. ∗ Evasive female behavior.

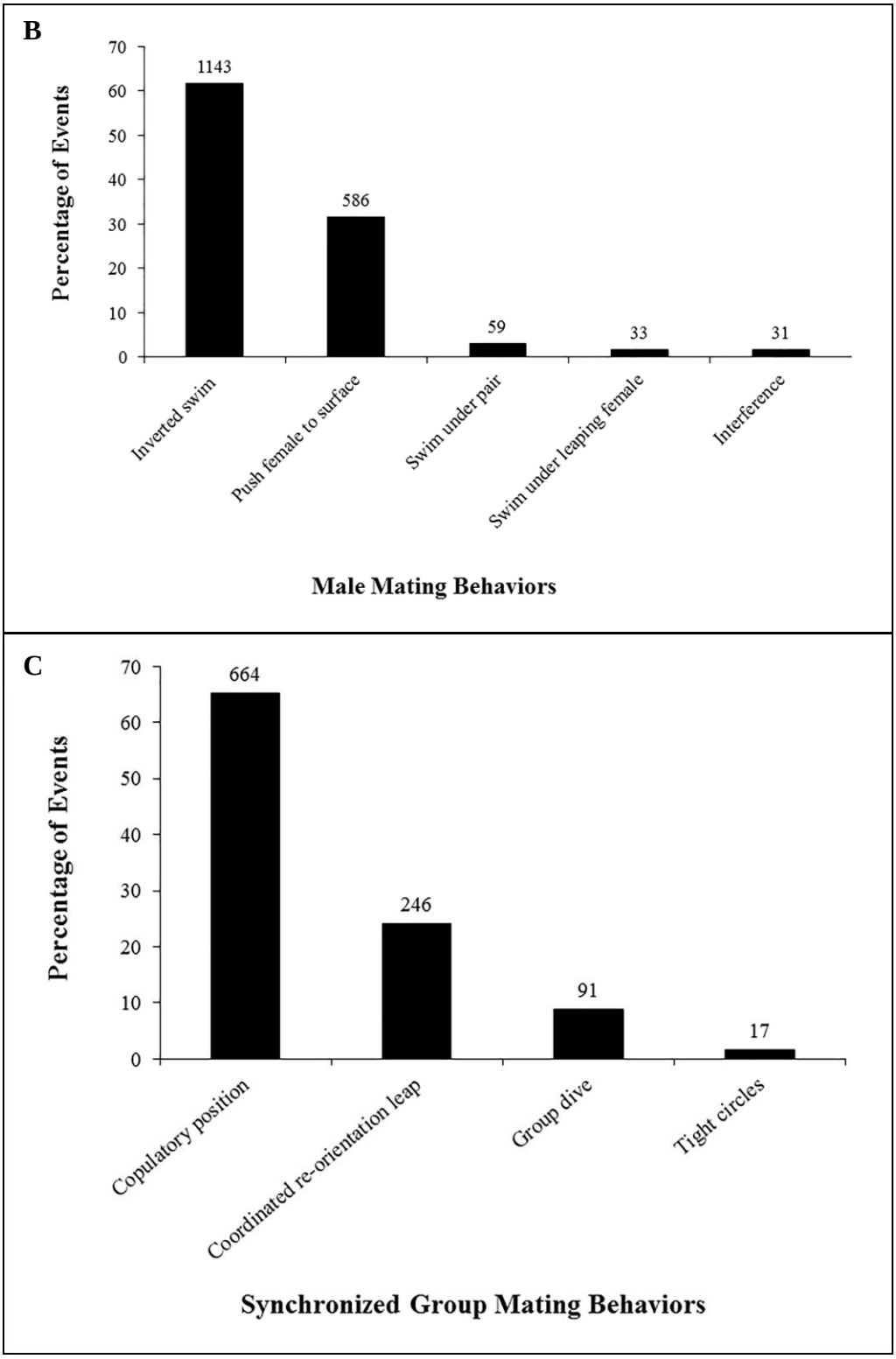

Figure 2-1, Continued.

rare. Only copulatory position fit our definition of non-evasive. The other three synchronous group behavior types consisted of evasive female behavior coordinated with one or more male dolphins.

Sequences of Behaviors Prior to Copulation

A total of 110 copulatory sequences were included in the analysis; each varied from 2 to 6 behavioral events. Females were resistant in 83% of copulatory sequences. The remaining non-resistant copulatory sequences were terminated by males (8%) or mutual actions of both the male and female (9%). The kinematic diagram for non-resistant copulatory sequences (Fig. 2-2) was less complex than for resistant copulatory sequences (female non-resistant: 17 types of adjacent events; female resistant: 37 types of adjacent events; Fig. 2-3). Group size was larger for resistant (mode: 5, SE: 0.5, range: 2 - 25) than non-resistant (mode: 3, SE 0.34, range: 2 - 8) copulatory sequences.

In both kinematic diagrams (Figs. 2-2, 2-3), the most frequent behavior type preceding the copulatory position was male push female to surface, which was preceded by male inverted swim. These two adjacent events characterize a typical chain of events in a 'normal' copulation sequence. All other adjacent events had simple probabilities below 0.1 when females were non-resistant. In the kinematic diagram for resistant copulatory sequences (Fig 2-3), female direction change also had simple probabilities above 0.1. Direction change was likely to follow inverted swim and precede push female to surface. Female behaviors were not successful in preventing copulatory position, as the body positioning was our criteria for inclusion in this analysis. There were three

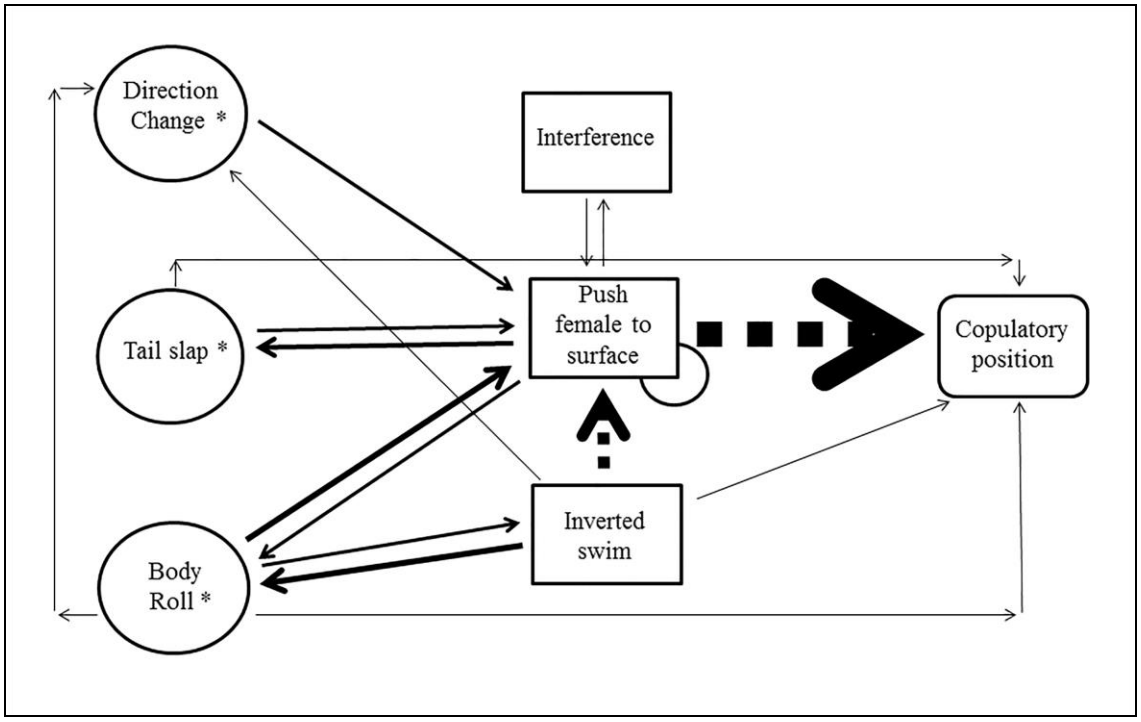

Figure 2-2. Kinematic diagram of female non-resistant copulatory sequences. The circles denote female behavior types, the rectangles denote male behavior types, and the rounded rectangles denote synchronized group behavior types. The thickness of each arrow represents its simple probability value. The dashed arrows indicate simple probabilities greater than 0.1. N = 71 adjacent events. ∗ Evasive female behavior.

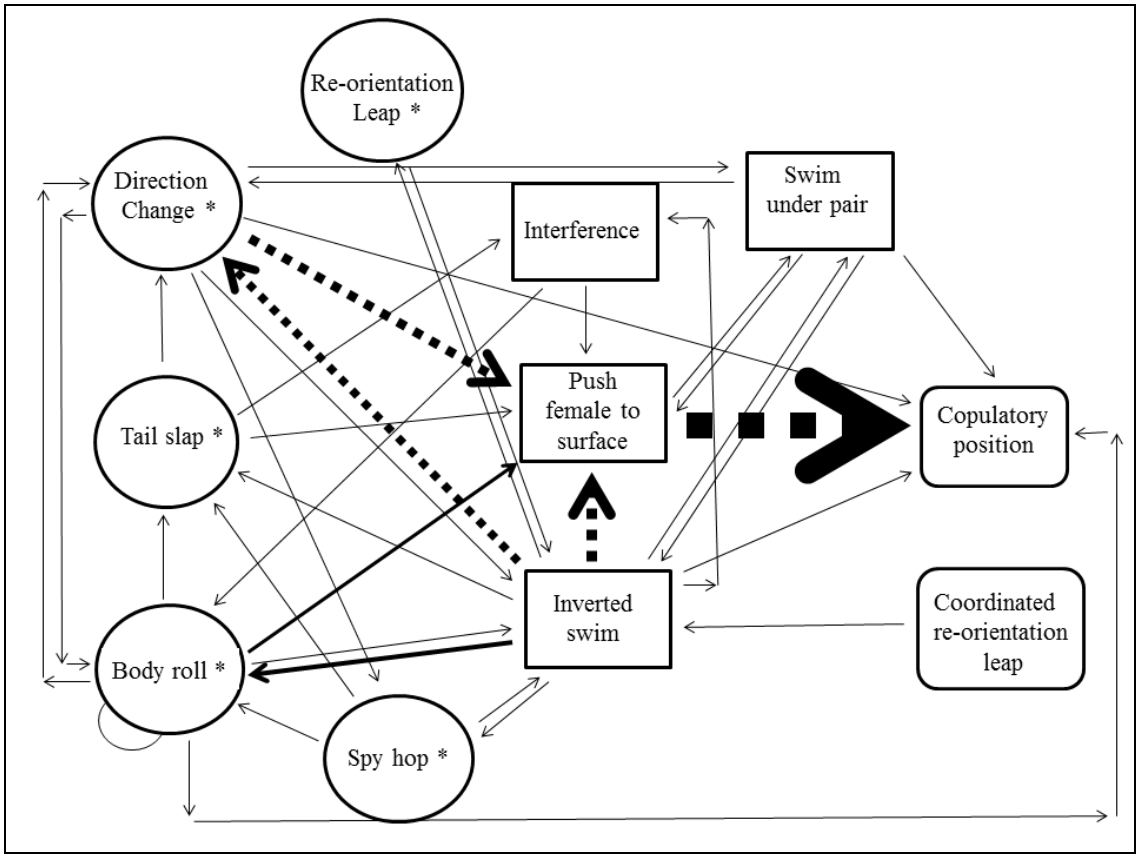

Figure 2-3. Kinematic diagram of female resistant copulatory sequences. The circles denote female behavior types, the rectangles denote male behavior types, and the rounded rectangles denote synchronized group behavior types. The thickness of each arrow represents its simple probability value. The dashed arrows indicate simple probabilities greater than 0.1. N = 342 adjacent events. ∗ Evasive female behavior.

times more male than female-initiated behaviors in both non-resistant and resistant copulatory sequences.

Female Responses to Male Signals

All female and all synchronized group responses to male behaviors throughout mating sequences are illustrated in Figure 2-4. The overall female response of the five target behavior types to the two most frequent male behavior types was significantly different than expected by chance (G^2_5= 217.80, N = 1472, p = 0.001; Fig. 2-4). Inverted swims were associated with body rolls (z = 6.62), direction changes (z = 1.96), re-orientation leaps (z = 8.14), and spy hops/speed bursts (z = 3.49). Push female to surface was associated with tail slap responses (z = 8.28). Females were no more likely to respond with copulatory position to the male signals of inverted swim (z = 0.06) compared to push female to surface (z = -0.06).

Discussion

We reject the 'non-evasive female' model for dusky dolphins near Kaikoura because copulation was the only non-evasive behavior shown by females in mating groups and females did not have random responses to male signals. We did not observe any affiliative behavior types like the rubbing, petting, and physical contact observed in other species of dolphins (e.g., Indo-Pacific bottlenose dolphins; Sakai et al., 2006; Atlantic spotted dolphins, *Stenella frontalis*; Dudzinski, 1998), although social contact is within the dusky dolphin behavioral repertoire (Markowitz, 2004, 2012). Although

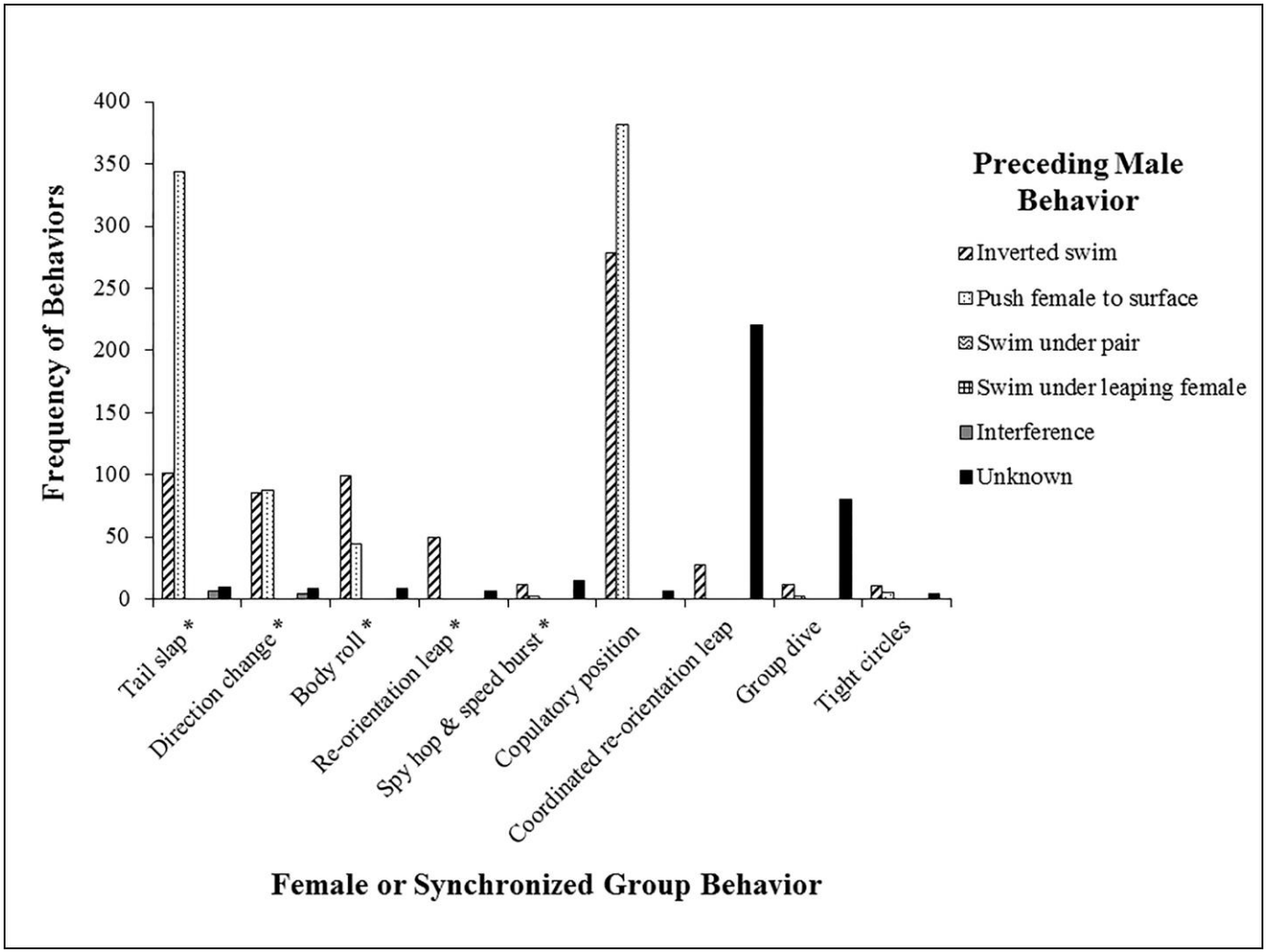

Figure 2-4. Frequencies of female and synchronized group mating behaviors immediately following male mating behaviors throughout mating follows. ∗ Evasive female behavior.

inactive behaviors, such as floating, were observed in non-mating contexts and in a mating context for other small delphinid species (pers. obs.), inactive behaviors were not recorded on video for mating groups. The behavioral repertoire size of female dusky dolphins was large compared to what has been reported for other species with male scramble competition for access to mates (i.e., fleeing: eastern grey squirrels, Koprowski, 1993, 2007; honey bees, *Apis mellifera*; Couvillon et al., 2010). However, the behaviors of females in other species with male scramble competition are largely unknown and should be more fully investigated.

Evasive behaviors occurred within copulatory sequences of both nonresistant ('normal') and resistant females, as indicated by the dolphin terminating the copulation. Female evasive behavior types were observed previous to this study at higher frequencies in mating than small adult non-mating and nursery groups near Kaikoura (Markowitz, 2004, 2012; Markowitz et al., 2010). The sequences of behavior types and their prevalence in facilitating or impeding copulation have not been previously reported. 'Normal' sequences involved a male approaching a female in inverted swim followed by pushing her to the surface prior to copulation. Resistant female sequences also included a direction change following inverted swims and preceding being pushed to the surface, suggesting direction changes are an important behavior unit for resistant females. High rates of direction changes have been associated with low rates of copulation (Markowitz et al., 2010).

In addition to the high occurrence probabilities of two adjacent events with a female behavior type, resistant females occurred in larger groups and had a more complex

structure of sequences compared to non-resistant females. Orbach et al. (2014) hypothesized that group sizes would be large and females would be evasive when they were close to ovulation and most attractive to males. An increased complexity in sequence structure could reflect differences in sample size between the two sequence categories, adaptability of dolphins to fluctuating social environments (e.g., responses to larger group sizes), requirements for increased manipulation when there is a conflict of interest between the sexes, or learned counter-tactics between sexes.

We present evidence that female behaviors following male behaviors were not random and fit the 'evasive female' conceptual model. We acknowledge that the mechanisms influencing female behavior associations with preceding male behaviors may be internally driven rather than evidence of cognitive processing of external information. From the proximate perspective, female evasive behaviors can indicate indirect mate choice (Wiley and Poston, 1996). We do not claim that non-random sequences are sufficient evidence for mate choice. Additional evidence needed to demonstrate female active mate choice include individual male identity, consistent individual variation in male mating behaviors, male variation in traits, and differential response of females to individual males that vary relative to the time of ovulation. These criteria were beyond the scope of this study and are challenging to test. Dusky dolphins are difficult to identify/follow as individuals while they are inverted during copulations, and are also difficult to follow (population size >10,000; Markowitz, 2004) throughout the breeding season or a lifetime.

From an ultimate perspective, evasive female behavior may function in 'evaluating' the vigor and endurance of prospective mates during extended chases (Whitehead and Mann, 2000) and favor those able to 'keep up' (Couvillon et al., 2010). In the absence of direct benefits from mating with a male (e.g., nuptial gifts), females may choose mates based on honest indicators of 'good genes' and the potential genetic quality of their offspring (Zahavi, 1975). For example, more vigorous male pronghorn (*Antilocapra americana*) were more attractive to females, and their sons were more viable than less vigorous males (Byers and Waits, 2006). Male dolphins capable of 'keeping up' with the unpredictable movements of females, such as abrupt changes in swimming direction or speed, may have extra copulation opportunities. Markowitz et al. (2010) suggested that the maneuverability and energy expended to successfully 'catch' a female dusky dolphin would also be advantageous when capturing prey and escaping predators.

Female evasive behaviors could have alternative non-mutually exclusive functions to mate rejection and evaluation. Like some primates (e.g., chimpanzees, bonobos, and spider monkeys) and other social and intelligent mammals, dolphins can exhibit behavioral plasticity in social and foraging strategies based on shifting costs and benefits of fission-fusion dynamics (Pearson, 2008; Kappeler et al., 2013; Würsig and Pearson, 2014). Alternative non-mutually exclusive hypotheses to account for the evasive tactics we documented might include (1) changes in female behaviors related to the estrous cycle, condition, and/or age (Cotton et al., 2006); (2) secluded consortships out of view by observers once a female has chosen a male; and/or (3) an observer effect

somehow interrupting courtship sequences. Future studies might address these information gaps. Ovulatory state has been determined via analyses of hormonal metabolites in urine or feces of cetaceans (Robeck et al., 1994; Rolland et al., 2005; Steinman et al., 2012). The deployment of animal-borne video cameras (e.g., Marshall et al., 2007) attached to dolphins could collect data on extended courtship sequences underwater at a distance from the observation vessel. We could detect no evidence of an observer effect (i.e., dolphins continued chasing and copulating in our presence). However, future studies using unmanned aerial vehicles to record mating groups could address questions about whether the presence of our research vessel influenced dolphin behavior.

Our observations exemplify how ecological conditions can drive behavior patterns. Prey is spatially and temporally reliable near Kaikoura, New Zealand (Dahood and Benoit-Bird, 2010). Predation pressure is reduced by large aggregation formations (Srinivasan and Markowitz, 2010). In this setting for low monopolization potential of mates, male dusky dolphins compete by exploitative scramble competition (Orbach et al., 2014) and do not cooperate (Orbach et al., 2015c). Male aggression towards females is low and there appear to be few direct costs to resistance (Orbach et al., 2015b). Accordingly, we observed high frequencies of female evasive behaviors in addition to complex structures of adjacent events in copulatory sequences. In contrast, for male Indo-Pacific bottlenose dolphins in Shark Bay, Western Australia, food is patchily distributed, predation pressure is high, and small group sizes predominate (Connor et al., 2000b). Males form small alliances and sequester females with aggressive herding

(Connor et al., 1992a, 1992b, 1996, 2000b). Males cooperate and position themselves on either side of the female to herd her (Connor et al., 1992a, 1992b). In this setting of high monopolization potential of mates, direct costs (e.g., physical injuries, reduced foraging opportunities, etc.) to females for evasion are high (Watson, 2005). Females are predicted to have low frequencies of evasive behaviors and less complex structure of adjacent events, which could reduce costs of sexual conflict in the aggressive consort setting.

We reject the 'non-evasive female' model for dusky dolphins near Kaikoura. Female behavioral repertoires consisted of more evasive than non-evasive behavior types, evasive behaviors were frequently exhibited, and females displayed non-random responses to male signals. The structure of copulatory sequences was more complex when females were resistant. Female evasive behaviors could have several non-mutually exclusive proximate and ultimate functions. Within the coevolutionary mating 'arms race', there may be a selective advantage for males with the endurance to remain close to females during mating chases, female ability to outmaneuver coercion by overly zealous suitors, and both sexes' ability to learn the consequences of their actions.

CHAPTER III

VARIATION IN FEMALE REPRODUCTIVE TRACT MORPHOLOGY OF THE COMMON BOTTLENOSE DOLPHIN (*TURSIOPS TRUNCATUS*)*

Summary

Cetaceans exhibit vaginal folds, unusual protrusions of the vaginal wall into the vaginal lumen. Inconsistent terminology and a lack of anatomical landmarks in the literature have hindered comparative studies of the form and function of vaginal folds. Our objectives are to: (1) develop a standardized measurement protocol for the reproductive tracts of female cetaceans, (2) assess variation in morphometrics within the common bottlenose dolphin (*Tursiops truncatus*), and (3) determine if vaginal muscle is skeletal, and therefore of somatic origin in this species. We selected 15 measurements to characterize female reproductive tracts and evaluated variability using fresh or frozen-thawed specimens from southeastern USA representing a range of sexual maturity states and reproductive states (n = 18 specimens). Presence of skeletal muscle and variation in the density of muscle banding were assessed using 90 histological samples (n = 5 specimens). Analyses of the gross morphological data revealed that the dolphins generally had one large vaginal fold that bisected the vaginal lumen. Vaginal

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morphology was similar for sexually mature and immature specimens and across reproductive states. The histological data revealed that the vaginal musculature consisted of smooth muscle, consistent with other mammals, leading us to conclude that vaginal contractions are likely under autonomic rather than somatic control. No differences were found in the density of smooth muscle banding among vaginal regions or sexual maturity states. Our systematic protocol lays the foundation for evaluating the function (e.g., sexual selection, natural selection) and evolution of vaginal folds.

Introduction

In many taxonomic groups, male genitalia have been preferentially described as compared to female genitalia (Ah-King et al., 2014). Most existing publications on the female genitalia of cetaceans (whales, dolphins, and porpoises) have focused on the ovaries that can be used to infer life history characteristics (Sljper, 1966; Harrison and Ridgway, 1971; Perrin et al., 1984; Marsh and Kasuya, 1986; Plön and Bernard, 2007; Dabin et al., 2008), or genital diseases and anomalies (Woodhouse and Rennie, 1991; Van Bressem et al., 2000; Van Elk et al., 2009). Few internal measurements of cetacean vaginal morphology have been routinely collected during necropsies over the past few decades.

The cetacean vagina contains unusual transverse fold(s), first described by Hunter (1787). These protrusions of the vaginal wall often occur in the cranial portion of the vagina, caudal to the cervix (Schroeder, 1990). Vaginal folds are most prominent on the dorsal vaginal wall and project into the lumen of the vagina, with the distal ends

directed towards the vulva (Pycraft, 1932). Various terms have been used to designate these cylindrical vaginal structures, including transverse rugae (Jackson, 1845), valvular folds (Murie, 1873), spermathecal folds (Meek, 1918), pseudo-cervices (Pycraft, 1932; Schroeder, 1990), circular folds (Ommanney, 1932; Green, 1977; Tarpley and Hillmann, 1999), vaginal folds (Morejohn and Baltz, 1972; Clarke et al., 1994), and rings of transverse folds (Chen et al., 1984). Across multiple species, these transverse vaginal folds have also been described as pleated, triangular-shaped, saucer-shaped, rugose, deep, leaf-like, crisscrossed, and puckered (Murie, 1873; Pycraft, 1932; Chen et al., 1984). Existing publications on the presence, numbers, shapes, and sizes of these folds are dated and inconsistent (Scott and Parker, 1889; Meek, 1918; Pycraft, 1932; Harrison, 1949; Morejohn and Baltz, 1972; Harrison and McBrearty, 1973; Schroeder, 1990; Woodhouse and Rennie, 1991; Clarke et al., 1994). For example, Meek (1918) reported two vaginal folds in the harbor porpoise (*Phocoena phocoena*), while Daudt (1898) reported nine to twelve folds in the same species. Morejohn and Baltz (1972) reported an absence of vaginal folds in immature Dall's porpoises (*Phocoenoides dalli*), because vaginal structures were not funnel-shaped, while the corresponding image showed numerous circular vaginal folds. When measurements have been provided, accompanying images and descriptions of anatomical landmarks have often been missing, which makes it difficult to reproduce these data (e.g., Murie, 1873). While some of the incongruence is likely due to species-specific diversity, standardized terminology and a protocol of measurements with clearly defined anatomical landmarks are necessary precursors to explore diversity and functionality.

The functions of cetacean vaginal folds are unclear and alternative functional hypotheses are not mutually exclusive (Clarke et al., 1994). It is most commonly hypothesized that the vaginal folds relate to mating in water, although their exact function is not clear and empirical tests of mechanisms are lacking. Vaginal folds may be adaptations to prevent the incursion of seawater into the upper reproductive tract once the penis is withdrawn (Slijper, 1962; Green, 1972; Green, 1977; Chen et al., 1984; Schroeder, 1990; Robeck et al., 1994). For example, the vaginal folds could "squeegee" seawater off the penis as it penetrates the cranial vagina (T. Robeck, pers. comm.). This hypothesis is supported by evidence that seawater is lethal to cetacean sperm (Schroeder and Keller, 1989). Vaginal folds have also been hypothesized to serve as vaginal plugs to prevent the loss of semen (Meek, 1918; Harrison, 1969). Retention of semen inside the female reproductive tract could be particularly important as male cetaceans lack the seminal vesicles and bulbourethral glands that typically facilitate coagulation of semen (Slijper, 1966; Harrison, 1969). Alternatively, the contact of vaginal folds with the penis could stimulate ejaculation (Meek, 1918; Harrison, 1969). The function of vaginal folds could also be associated with pregnancy rather than mating. For example, the folds could prevent miscarriages during pressure changes while diving (Kellogg, 1938) or distend during parturition and ease the birthing process (Meek, 1918; Slijper, 1962). Similar folds have been reported in some closely related terrestrial artiodactyls (e.g., domestic pigs, *Sus domesticus*; Dyce et al., 2010), potentially indicating that vaginal folds are not an adaptation to an aquatic environment and could be inherited through a common ancestor (Pabst et al., 1998).

Based on what little evidence exists, cetacean vaginal folds are muscular and capable of contractile movement (Harrison, 1949; Chen et al., 1984). The ability to contract may help elucidate a potential function. Harrison (1949) hypothesized that vaginal folds engage in a pumping action that directs seminal fluids cranially towards the uterine horns where fertilization occurs. Alternatively, the pumping action of vaginal muscles could shunt sperm caudally away from the uterine horns. For example, in both rats and cats, uterine contractions are propagated in both cranial and caudal directions, with caudal-oriented peristalsis expected to carry sperm away from the uterine horns (Crane and Martin, 1991; Chatdarong et al., 2002). The vaginal folds of cetaceans project caudally towards the vaginal opening, potentially facilitating the caudal direction of sperm expulsion. In diverse terrestrial taxonomic groups, females can selectively reject sperm by shunting it within their reproductive tracts away from sites of fertilization (Eberhard, 1996; Dean et al., 2011; feral domestic fowl, *Gallus gallus domesticus*; Pizzari and Birkhead, 2000; arctiid moths, *Utetheisa ornatrix*; Curril and LaMunyon, 2006; Grevy's zebras, *Equus grevyi*; Ginsberg and Rubenstein, 1990). Sexual selection by cryptic female choice entails females biasing paternity after copulation that can favor particular mates (e.g., those with genital designs that elicit more effective stimulation during copulation), and is particularly prevalent in species in which females mate with multiple males and have complex reproductive morphology (Eberhard, 1996, 2010). Anatomical analyses of vaginal gross morphology and musculature are necessary first steps to test the potential function(s) of vaginal folds in

sexual selection and natural selection before distinguishing between active uptake or expulsion of semen, or no active semen movement.

The mammalian vaginal wall consists of the internal mucosal/submucosal layer (epithelium/connective tissue), an intermediate muscularis layer (smooth muscle), and an external adventitial layer (connective tissue) (Coleman, 2001). Skeletal muscles (e.g., *M. bulbospongiosus*) are located near the vaginal wall and are part of the urogenital diaphragm (Oelrich, 1983). Unlike smooth muscle, which is under autonomic control, skeletal muscle is under somatic control. Skeletal muscle can be distinguished from smooth muscle by the presence of striations. If skeletal musculature is present in the vaginal folds of cetaceans, unlike most other mammals that lack vaginal folds, the musculature could provide females with a mechanism to pump the sperm of select males within their cranial vaginal tract. However, to our knowledge, skeletal muscle has not been described in the muscularis region of any mammalian vaginal wall. Past studies of the microstructure of cetacean vaginal folds are sparse and have focused on the mucosal layer (Tarpley and Hillmann, 1999) rather than the muscularis layer. Microstructural modifications within the female reproductive tract can also reflect functions, such as expansion during parturition or copulation (e.g., spotted hyenas, *Crocuta crocuta*; Cunha et al., 2003). Among cetaceans, Harrison (1949) reported that long-finned pilot whales (*Globicephala melas*) had smaller, more numerous, and closely-spaced muscle bundles within their caudal vaginal walls compared to their cranial vaginal folds.

Our study was designed to characterize the gross morphology of reproductive tracts of female common bottlenose dolphins (*Tursiops truncatus*) and assess whether

vaginal muscle tissue is skeletal. Our approach to addressing these questions included development of a standardized protocol with anatomical landmarks to collect measurements of reproductive tract morphology. We validated the reliability of the protocol by exploring variation across sexual maturity states, reproductive states, and geographic areas. We present the protocol in detail here to facilitate future comparative studies. We also characterized muscle types and the density of muscle bands in vaginal tissue.

Materials and Methods

Specimen Collection

Female reproductive tracts of common bottlenose dolphins were collected from opportunistic beach strandings along the southeastern U.S. coastline. Specimens were provided by marine mammal stranding networks located in Galveston, Texas, Morehead City, North Carolina, Jacksonville, Florida, and Virginia Beach, Virginia. Tissues were collected from fresh (< 24 hours post-mortem) or moderately decomposed deceased animals. Intact reproductive tracts, from the external uro-genital slit through to the ovaries, were obtained. Specimens used for gross morphological measurements were analyzed while fresh or were frozen as soon as possible and transferred to facilities located at Texas A&M University at Galveston. Separate specimens were used for the histological analysis that were collected exclusively from Texas and were formalin-fixed while fresh. One specimen was used for both gross morphological and histological analyses (Table 3-1). For this specimen, gross morphological measurements were

Table 3-1. Count of the number of common bottlenose dolphin specimens divided by geographic area and state of sexual maturity. Within the sexually mature state, specimens are further divided into reproductive states. Within the geographic area columns, specimens from Texas are divided into those used for gross morphological (top row) or histological (bottom row) analysis. Different specimens were used for gross morphological and histological techniques with the exception of one specimen denoted with an * symbol.

State of Sexual Maturity	Reproductive State	Geographic Area			
		Texas	Florida	North Carolina	Virginia
Mature	Pregnant	2 1*	0	1	0
	Lactating	1 1	2	0	0
	Resting	3 1	3	2	1
Immature		0 2	0	2	1

collected while the tissue was fresh and prior to formalin-fixation. Information about each specimen was provided by the stranding network that collected the animal, including the date, location, and degree of decomposition at the time of stranding. In addition, total body length, state of sexual maturity (based on the presence of *corpora lutea* and/or *corpora albicantia* on either ovary, or body length), and reproductive state (i.e., pregnant, lactating, or resting) data were provided. All reproductive tracts were collected under a National Marine Fisheries Service (NMFS) Southeast Regional Office salvage permit letter to one of the authors (D.N.O.).

Gross Morphological Measurements

Up to fifteen measurements were recorded for fresh or frozen-thawed specimens. Anatomical landmarks and measurements are shown in Figure 3-1. All measurements were collected with the excised reproductive tracts oriented in dorsal recumbency (ventrum-up). Each ovary was assessed for the absence or presence of *corpora lutea* and/or *corpora albicantia* (Ivashin, 1984). The specimens were bisected by a ventral incision along the longitudinal midline of the reproductive tract. The incision was made from the external clitoris through to the internal bifurcation of the uterine horns (Fig. 3-1). The uterine horns were opened longitudinally and their appearance was characterized as either longitudinally banded (Fig. 3-2) or non-banded in texture (Fig. 3-3). Measurements were consistently collected with the specimens in a relaxed state rather than stretched out.

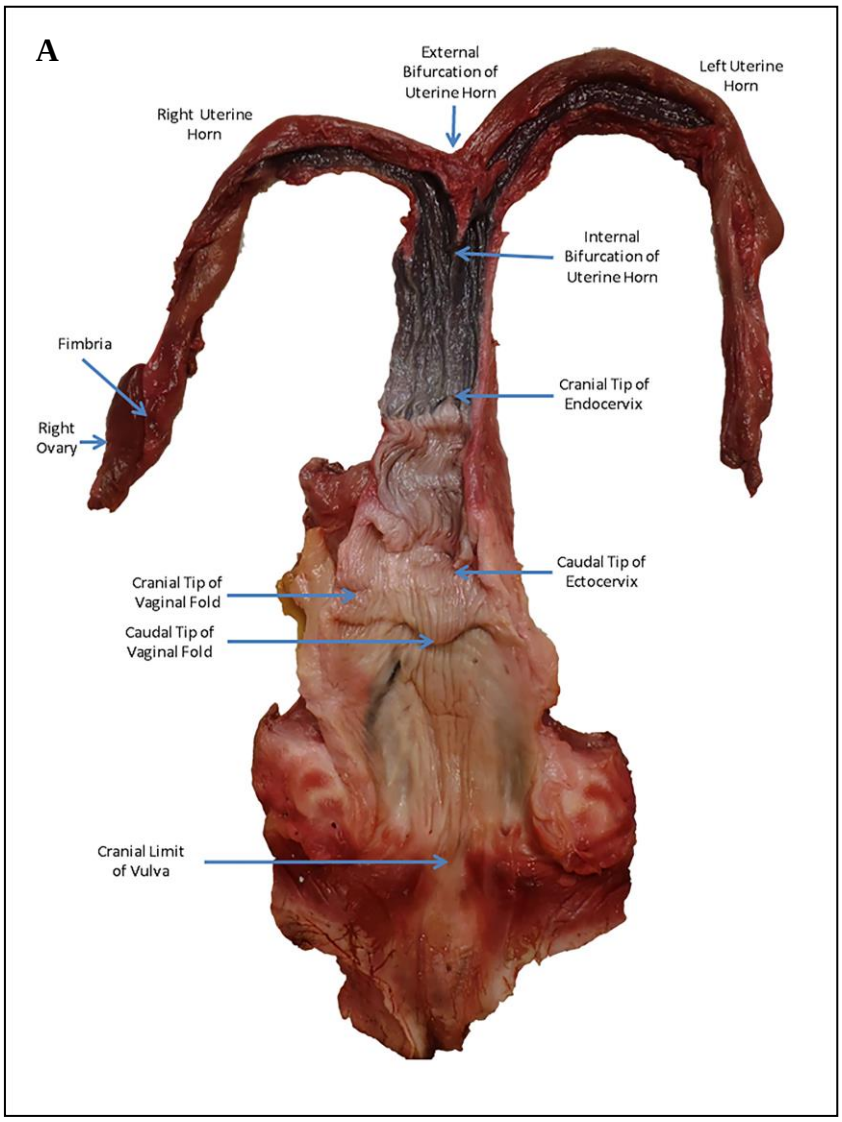

Figure 3-1. Dissected reproductive tract of a female common bottlenose dolphin with labels of (A) morphological landmarks and (B) measurements. The frozen-thawed specimen is oriented in dorsal recumbency. The specimen was splayed open, but the tissue was not stretched for any measurements. The right, but not the left ovary is included in this specimen. The arrows delineate the start and end points of the individual measurements. Curvilinear lengths are distinguished with an asterisk (*) symbol. Dashed lines denote measurements on the underside of tissue not visible in the figures. Width measurements in transverse plane correspond with the circumference of the lumens straightened out.

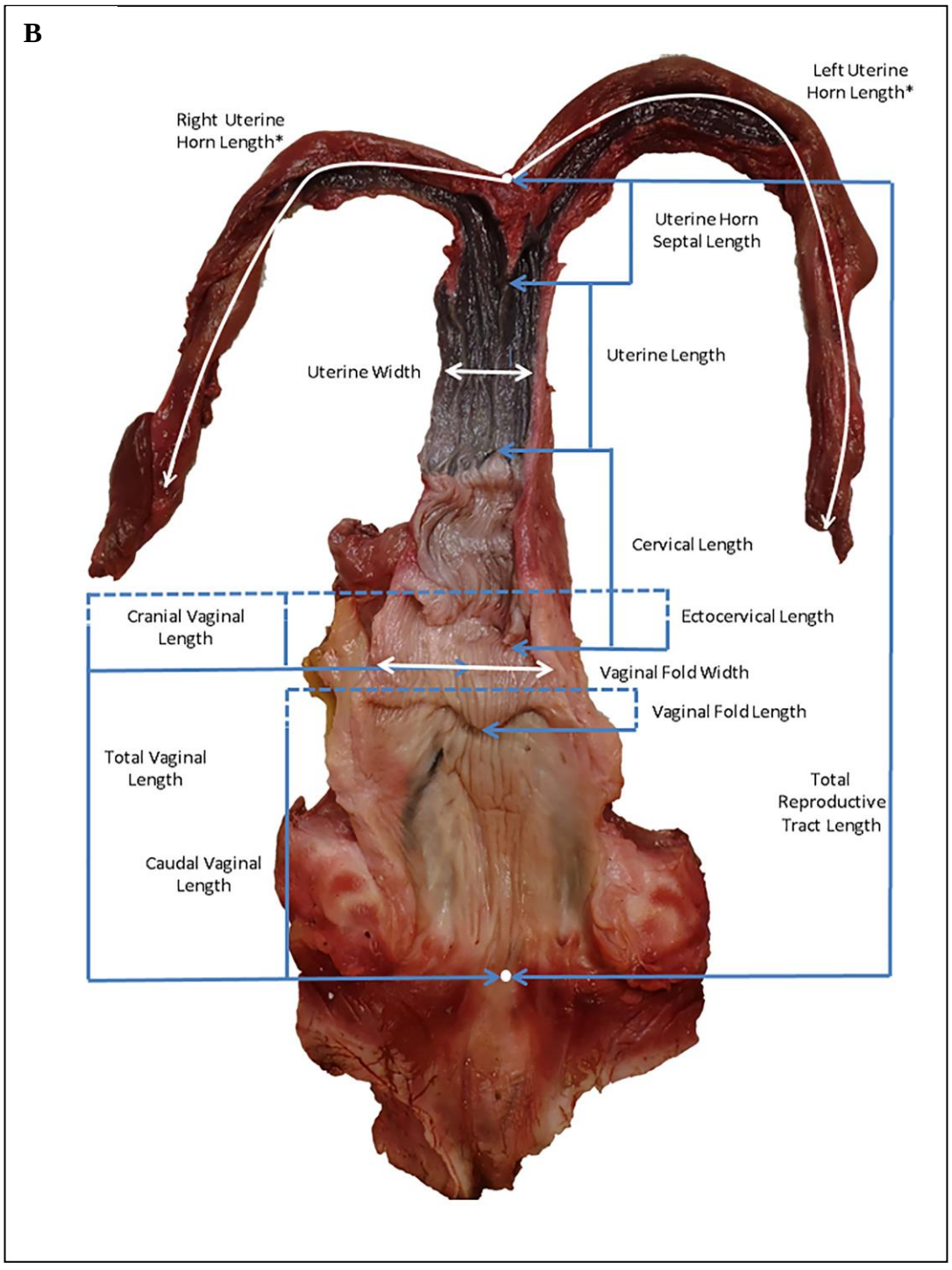

Figure 3-1, Continued

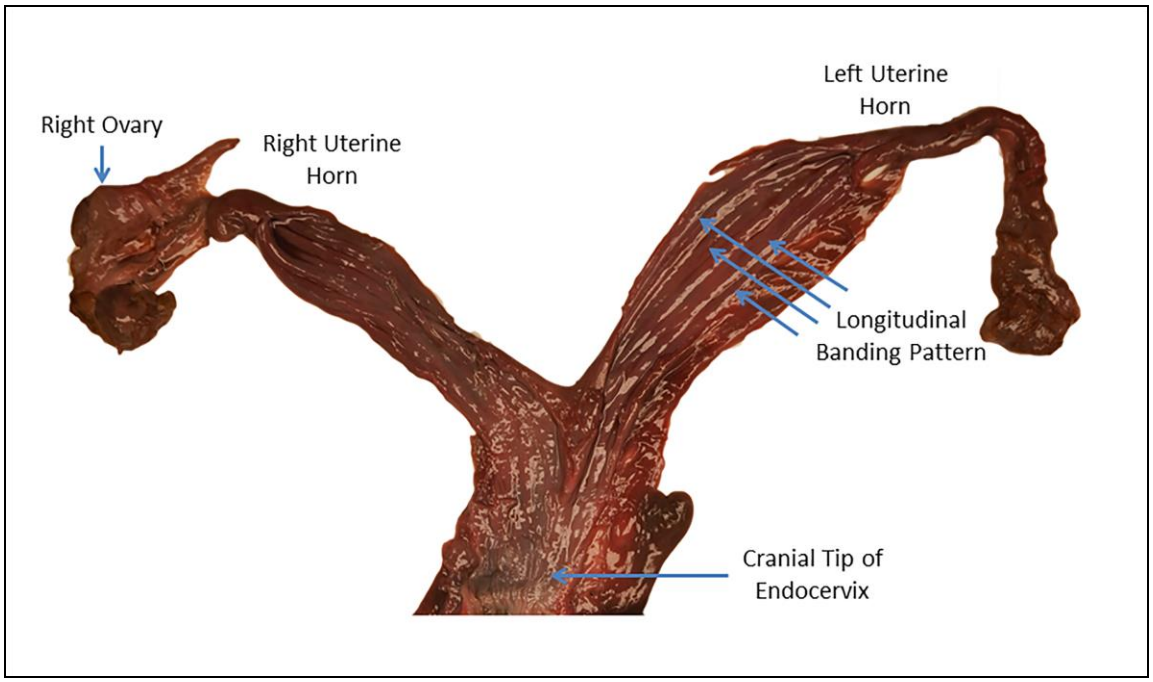

Figure 3-2. Uterine horns and uterus of a sexually immature female common bottlenose dolphin. The frozen-thawed specimen is oriented in dorsal recumbency. The uterine horns and uterus were opened longitudinally. The specimen was splayed open, but the tissue was not stretched. The right, but not the left ovary was included in this specimen. The internal tissue of the uterine horns had a banded texture pattern along its longitudinal axis that gave it a striped appearance. The uterine horn and uterus tissue were not distended.

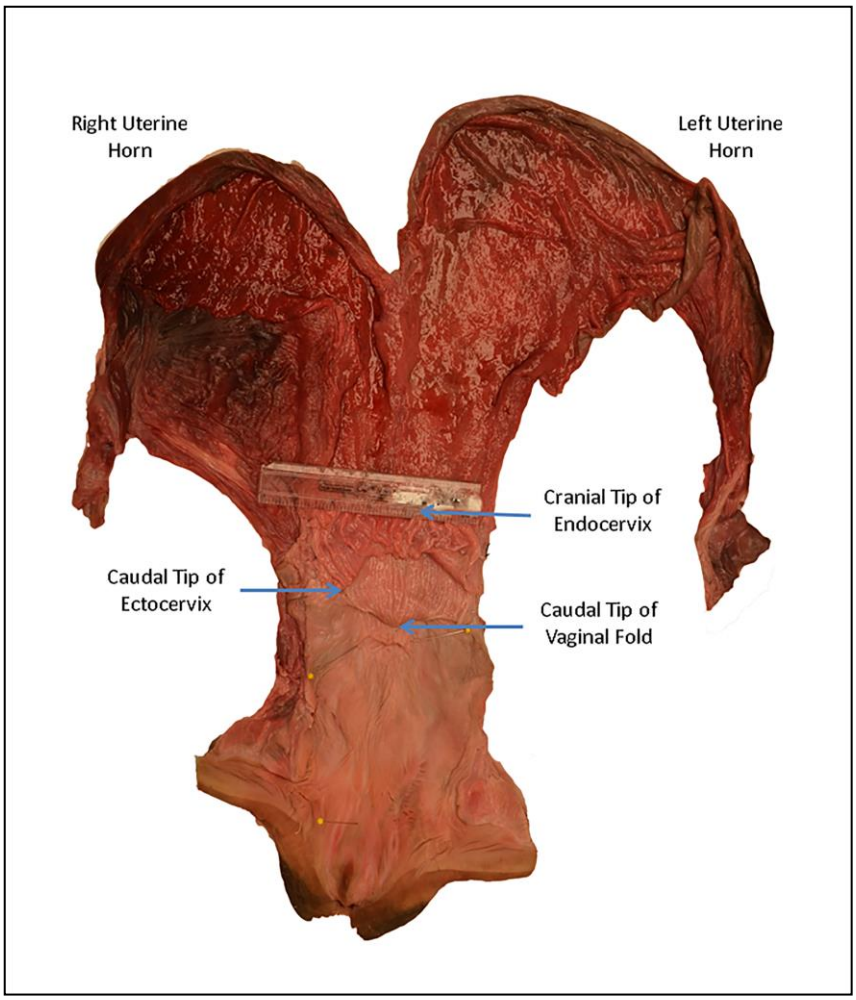

Figure 3-3. Dissected reproductive tract of a sexually mature female common bottlenose dolphin. The frozen-thawed specimen is oriented in dorsal recumbency. The uterine horns and entire reproductive tract were opened longitudinally. The specimen was splayed open, but the tissue was not stretched. The uterine horns were wide in diameter proximal to the uterus and tapered off in length closer to the ovary. The internal tissue of the uterine horns did not have a banded texture pattern along its longitudinal axis. The bottom of the ruler demarcates the cranial limit of the cervix. The cranial and caudal limits of the cervix were serrated and uneven. The vaginal fold was located immediately caudal to the cervix in the transverse plane and spanned the entire width of the vaginal canal. Two yellow pins demarcate a shallow transverse ridge in the lateral plane caudal to the vaginal fold.

Measurements of the upper reproductive tract (Table 3-2) included the:

- Left and Right Uterine Horn Lengths: from the cranial external bifurcation of the uterine horns to the distal *fimbria;* the oviduct was not measured separately from the uterine horn, because the point of delineation was unclear in some specimens
- Uterine Horn Septal Length: cranial-caudal length of the common median wall along the external bifurcation to the internal bifurcation of the uterine horns
- Uterine Length: cranial-caudal length from the internal bifurcation of the uterine horns to the cranial tip of the endocervix
- Uterine Width: in transverse plane; measured midway between the internal bifurcation of the uterine horns and the cranial tip of the endocervix. The cylindrical tube was bisected and spread open
- Total Reproductive Tract Length: cranial-caudal length from the external bifurcation of the uterine horns to the cranial limit of the vulva (delineated by the distal position of the *glans clitoridis*). We did not add uterine horn lengths to the total reproductive tract lengths, because of discrepancies between left and right horn lengths

Measurements of the lower reproductive tract (Table 3-2) included the:

- Cervical Length: cranial-caudal length from the cranial tip of the endocervix to the caudal tip of the ectocervix (*portio vaginalis*)
- Ectocervical Length: cranial-caudal length of the ectocervix from its base (where it met the dorsal vaginal wall) outward to its distal tip that projected into the vaginal lumen; measurement was on the dorsal side of the ectocervix

- Total Vaginal Length: cranial-caudal length from the base of the ectocervix (where it met the vaginal wall) to the cranial limit of the vulva
- Cranial Vaginal Length: cranial-caudal length from the base of the ectocervix (where it met the vaginal wall) to the cranial tip of the primary vaginal fold (the fold with the greatest projection into the vaginal lumen)
- Caudal Vaginal Length: cranial-caudal length from the base of the primary vaginal fold (where it met the vaginal wall) to the cranial limit of the vulva; measurement was on the dorsal side of the tissue
- Vaginal Fold Width: in the transverse plane; the width of the vaginal fold at its cranial end where it met the vaginal wall. The cylindrical tube was bisected and spread open
- Vaginal Fold Length: cranial-caudal length of the primary vaginal fold from its base (where it met the dorsal vaginal wall) outward to its distal tip that projected into the vaginal lumen; measurement was on the dorsal side of the vaginal fold
- Vaginal Fold Width-to-Vaginal Width Ratio: in transverse plane; ratio of vaginal fold width divided by total vaginal width. Vaginal fold widths and total vaginal widths were measured along the cranial line where the two structures met
- Number of Vaginal Folds: counted as those exceeding 5 mm in vaginal fold length

Table 3-2. Measurements of reproductive tract variation in common bottlenose dolphins. Mean (± SD) of gross morphological measurements collected from the reproductive tracts of up to eighteen female bottlenose dolphins. The sample size for the measurement is italicized below the mean. All length and width measurements are in mm. Values are raw data and are not scaled by body length.

	Sexual Maturity State		**Reproductive State (Sexually Mature Only)**			**Geographic Area (Sexually Mature Only)**			
Measurement	Mature	Immature	Pregnant	Lactating	Resting	Texas	Florida	North Carolina	Virginia
Total Body Length	2512.87 (± 182.18) *15*	1763.33 (± 587.31) *3*	2590.00 (± 314.80) *3*	2500.00 (± 219.31) *3*	2491.44 (± 135.92) *9*	2436.67 (± 170.37) *6*	2484.00 (± 160.87) *5*	2636.67 (± 197.57) *3*	2473.00 (± 0) *1*
Left Uterine Horn Length	510.57 (± 218.42) *7*	119.75 (± 33.59) *2*	792.50 (± 228.40) *2*	N/A *0*	397.80 (± 53.70) *5*	710.00 (± 3457) *2*	382.00 (± 50.91) *2*	463.33 (± 150.48) *3*	N/A *0*
Right Uterine Horn Length	375.88 (± 144.85) *8*	94.00 (± 38.18) *2*	595.00 (± 24.04) *2*	273.00 (± 79.20) *2*	317.75 (± 55.72) *4*	452.50 (± 177.48) *2*	265.67 (± 57.42) *3*	435.00 (± 158.29) *3*	N/A *0*
Uterine Horn Septal Length	73.84 (± 52.14) *13*	27.05 (± 10.00) *3*	236.12 (± 0) *1*	54.66 (± 16.23) *3*	62.20 (± 20.74) *9*	94.60 (± 79.73) *5*	54.03 (± 24.60) *5*	79.32 (± 18.70) *2*	58.12 (± 0) *1*
Uterine Length	57.74 (± 29.41) *13*	16.58 (± 13.59) *3*	148.87 (± 0) *1*	52.05 (± 11.22) *3*	49.17 (± 11.72) *9*	68.03 (± 46.25) *5*	52.61 (± 14.59) *5*	52.94 (± 1.20) *2*	41.50 (± 0) *1*
Uterine Width	86.69 (± 70.18) *14*	27.27 (± 11.14) *3*	238.30 (± 44.83) *2*	44.75 (± 11.01) *3*	66.99 (± 29.565) *9*	92.84 (± 73.76) *5*	62.19 (± 25.07) *5*	130.78 (± 120.60) *3*	46.17 (± 0) *1*
Total Reproductive Tract Length	272.67 (± 53.54) *9*	148.69 (± 51.62) *3*	305 (± 0) *1*	237.00 (± 7.07) *2*	279.17 (± 61.71) *6*	288.40 (± 56.11) *5*	250.01 (± 61.05)) *3*	/ *0*	262.00 (± 0) *1*
Cervical Length	48.77 (± 11.97) *14*	31.46 (± 10.50) *3*	59.21 (± 15.74) *2*	50.86 (± 13.01) *3*	45.75 (± 10.98) *9*	48.75 (± 12.37) *5*	43.13 (±1 1.13) *5*	59.62 (± 10.61) *3*	44.48 (± 0) *1*

Table 3-2, Continued.

	Sexual Maturity State		Reproductive State (Sexually Mature Only)			Geographic Area (Sexually Mature Only)			
Measurement	Mature	Immature	Pregnant	Lactating	Resting	Texas	Florida	North Carolina	Virginia
Ectocervical Length	32.36 (± 7.02) *14*	13.33 (± 6.03) *3*	38.50 (± 6.36) *2*	24.67 (± 4.51) *3*	33.56 (± 6.06) *9*	36.80 (± 5.67) *5*	28.80 (± 8.53) *5*	31.00 (± 5.20) *3*	32.00 (± 0) *1*
Total Vaginal Length	154.26 (± 35.03) *9*	89.67 (± 20.18) *3*	201.00 (± 0) *1*	119.01 (± 12.80) *2*	158.21 (± 31.38) *6*	171.16 (± 33.94) *5*	132.51 (± 31.55) *3*	/ *0*	134.98 (± 0) *1*
Cranial Vaginal Length	28.57 (± 11.76) *12*	18.31 (± 9.35) *3*	52.83 (± 0) *1*	29.29 (± 9.68) *3*	25.26 (± 9.69) *8*	36.04 (± 11.43) *5*	28.12 (± 8.27) *4*	20.25 (± 4.15) *2*	9.65 (± 0) *1*
Caudal Vaginal Length	111.23 (± 27.07) *9*	67.97 (± 8.36) *3*	152.00 (± 0) *1*	87.92 (± 5.42) *2*	112.20 (± 24.81) *6*	123.83 (± 27.87) *5*	94.37 (± 21.85) *3*	/ *0*	98.78 (± 0) *1*
Vaginal Fold Width	91.94 (± 30.62) *14*	39.30 (± 11.37) *3*	130.24 (± 32.44) *2*	69.38 (± 16.64) *3*	90.94 (± 27.64) *9*	86.00 (± 29.03) *5*	90.57 (± 31.41) *5*	113.92 (± 34.40) *3*	62.52 (± 0) *1*
Vaginal Fold Length	27.63 (± 5.78) *15*	9.00 (± 2.65) *3*	28.46 (± 6.66) *3*	28.33 (± 5.03) *3*	27.11 (± 6.35) *9*	24.06 (± 4.68) *6*	28.80 (± 5.76) *5*	33.33 (± 4.62) *3*	26.00 (± 0) *1*
Vaginal Fold Width-to-Vaginal Width Ratio	1.00 (± 0) *15*	1.00 (± 0) *3*	1.00 (± 0) *3*	1.00 (± 0) *3*	1.00 (± 0) *9*	1.00 (± 0) *6*	1.00 (± 0) *5*	1.00 (± 0) *3*	1.00 (± 0) *1*
Number of Folds	1.07 (± 0.26) *15*	1.33 (± 0.58) *3*	1.00 (± 0) *3*	1.33 (± 0.58) *3*	1.00 (± 0) *9*	1.00 (± 0) *6*	1.20 (± 0.45) *5*	1.00 (± 0) *3*	1.00 (± 0) *1*

Small transverse ridges (< 5 mm long from their base to distal tip) were counted and their distances from other vaginal landmarks were recorded. Transverse ridges were not included in subsequent analyses, because they would not likely impede the penis, were not deep crypts to trap or store sperm, and some were so fine the distinction between a ridge and smooth vaginal tissue was unclear in some specimens. With the exception of the left and right uterine horn lengths, ectocervical lengths, and vaginal fold lengths, all data were collected with digital calipers. The left and right uterine horn lengths were measured with dental floss as a curvilinear line. The ectocervical and vaginal fold lengths were collected with a clear plastic ruler positioned at the base (the lateral attachment at the fornix) of the structure (where it met the vaginal wall), to its distal tip. Additionally, measurements were taken down the longitudinal midline of the vagina except for the cervical length, ectocervical length, and vaginal fold length. These three measurements could be offset from the midline and were measured to the distal tips of the anatomical landmarks.

We established baseline reproductive tract measurements for female common bottlenose dolphins across sexual maturity states, reproductive states, and geographic areas (Table 3-1). The specimens were categorized as sexually mature or immature based on visual assessment of their ovaries. When ovaries were not available for examination, published region-specific asymptotic body lengths for maturity were used instead (Mead and Potter, 1990; Fernandez and Hohn, 1998; Mattson et al., 2006; Mallette et al., 2016). Only mature dolphins were used in the reproductive state and geographic area analyses, while immature and mature dolphins were used in the sexual

maturity state analysis. Mature animals were subcategorized as pregnant, lactating, or resting (non-pregnant and non-lactating) based on data provided by the stranding networks (i.e., fetus present or milk in mammary gland). To account for potential confounding effects associated with reproductive state, we conducted analyses for sexual maturity classes and geographic areas using (1) only sexually mature resting dolphins, and (2) all sexually mature animals. Reproductive tract measurements were standardized by total body lengths for comparisons across specimens. When reproductive tracts were incomplete, some measurements could not be collected (e.g., measurements of the total reproductive tract length, total vaginal length, and caudal vaginal length were not included in the analysis if the vulva was missing). Accordingly, there are inconsistent numbers of measurements for each specimen (Table 3-2). Due to our small and inconsistent sample sizes, and the large number of outliers (based on the 1.5x interquartile range rule), statistical analyses were not performed. The data scaled by total body lengths are presented as Tukey's box and whisker plots.

Histology

Specimens collected from Galveston, Texas, were preserved in ten percent physiologically-buffered formaldehyde for histological processing. Two samples (~ 6.5 cm^2) of cranial vaginal fold tissue were collected from each specimen. Two similarly-sized samples of (non-fold) vaginal tissue were collected along the same longitudinal plane, caudal to the region of vaginal folds. The tissues were fixed, dehydrated, cleared, and infiltrated with paraffin wax under vacuum. Tissues were then embedded in paraffin

blocks, and cut longitudinally at 7-10 µm on a Leica 2235 RM rotary microtome. Samples were collected from the muscularis layer of the tissue, since other regions of the vaginal wall have not been reported to contain muscle (Coleman, 2001). The resulting ten sections per sample were mounted on gelatin-coated slides and stained with a modified Masson's trichrome stain (Masson, 1929) following Marshall et al. (2006) to visualize the muscle tissue. Muscle fascicles were stained pink, connective tissues were stained green-blue, and cell nuclei were stained black (Luna, 1968). A total of forty slides (ten per sample) were prepared for each specimen.

To examine the microstructure of the vaginal tissue, we selected five slides at random from each tissue sample. Micrographs were collected using a Diagnostic Instrument Spot Pursuit camera fitted to a Nikon Eclipse E400 light microscope and SPOT Advanced Image software. One micrograph was collected per slide at 20x magnification. We imaged portions of the slides in which no other structures were present (fat cells, blood vessels, etc.), muscle bands (fascicles) were parallel and in the same plane, and muscle bands occupied a minimum width of 2 units on the reticle at 20x magnification. No adjustments besides the additions of scale bars, contrast, and brightness were made to the micrographs. The microstructure of the vaginal tissues was assessed in Image J (v.1.44p) by three researchers unaware of the tissue location (cranial vaginal fold or caudal vaginal wall tissue).

The presence or absence of smooth and skeletal muscle bands were evaluated visually, using reference images in Geneser (1985) and Hammersen (1985) as guides. Differences in the density of muscle banding patterns were compared between cranial

vaginal fold and caudal vaginal tissue. A diagonal line was placed across each image and the diagonal length was measured. Then the number of muscle bands that were crossed by the diagonal line and the sum of their collective thicknesses along the diagonal line were tallied. The density of muscle banding was derived by dividing the sum of muscle thicknesses by the length of the diagonal line. The average percent density of muscle banding was calculated per sample when the measurements of the three independent researchers were within 5 units (%) of each other. If the intermediate measurement was within 5 units of the upper and lower measurements, but the span of the three measurements was >5 units, the average of the two measurements with the smallest difference was used. Samples that resulted in >5 units discrepancies between the researchers were considered ambiguous and excluded from statistical analysis. A mixed model repeated measure Analysis of Variance test (with animal identity as the random variable) was used to determine if there was a difference in the density of muscle banding between vaginal fold and vaginal tissue, sexually mature or immature specimens, and the interaction between the tissue type and sexual maturity state (Stata, ver. 14, Stata Corp, College Station, TX). The predicted standardized residuals of the final model were evaluated for normality using quantile normal plots. Data with non-normal distributions (based on Shapiro-Wilk tests) were transformed to meet assumptions of normality (based on the best transformation method; Ladder test, Stata®) and reanalyzed. Pairwise comparisons of estimated marginal means were conducted with a Šidák adjustment to control for type 1 errors ($p < 0.05$).

Results

Gross Morphological Measurements

A suite of 15 reproductive tract measurements was obtained for 18 specimens representing different sexual maturity states, reproductive states, and geographic areas (Tables 3-1, 3-2). The general characteristics of the reproductive tracts are described. Color pigmentations throughout the reproductive tract varied with specimens and related to decomposition (Figs. 3-1 through 3-4). The ovaries were oval-shaped (Figs. 3-1, 3-2) and every sexually mature specimen had more *corpora lutea* and/or *corpora albicantia* in the left than right ovary. In sexually immature specimens, the left and right uterine horns were small in diameter and marked internally with fine longitudinal bands that gave the tissue a striped appearance (Fig. 3-2). In sexually mature specimens, the left and right uterine horns were greater in diameter and not banded internally, because the tissue was comparatively distended (Fig. 3-3). In pregnant females, both uterine horns were greatly distended. The distal tips of the ectocervix and endocervix were uneven and serrated in shape (Figs. 3-1, 3-3). The cervix contained fine internal longitudinal textured bands (Figs. 3-1, 3-3). Longitudinal bands were particularly prevalent on the endocervix and ectocervix (Fig. 3-1). Thick and sticky cervical mucus was congealed within the cervical cavity of many specimens, although the mucus sometimes extended into the vaginal lumen in a more diluted consistency. The ectocervix created a deep fornix in the cranial vagina that had the greatest length on the dorsal wall.

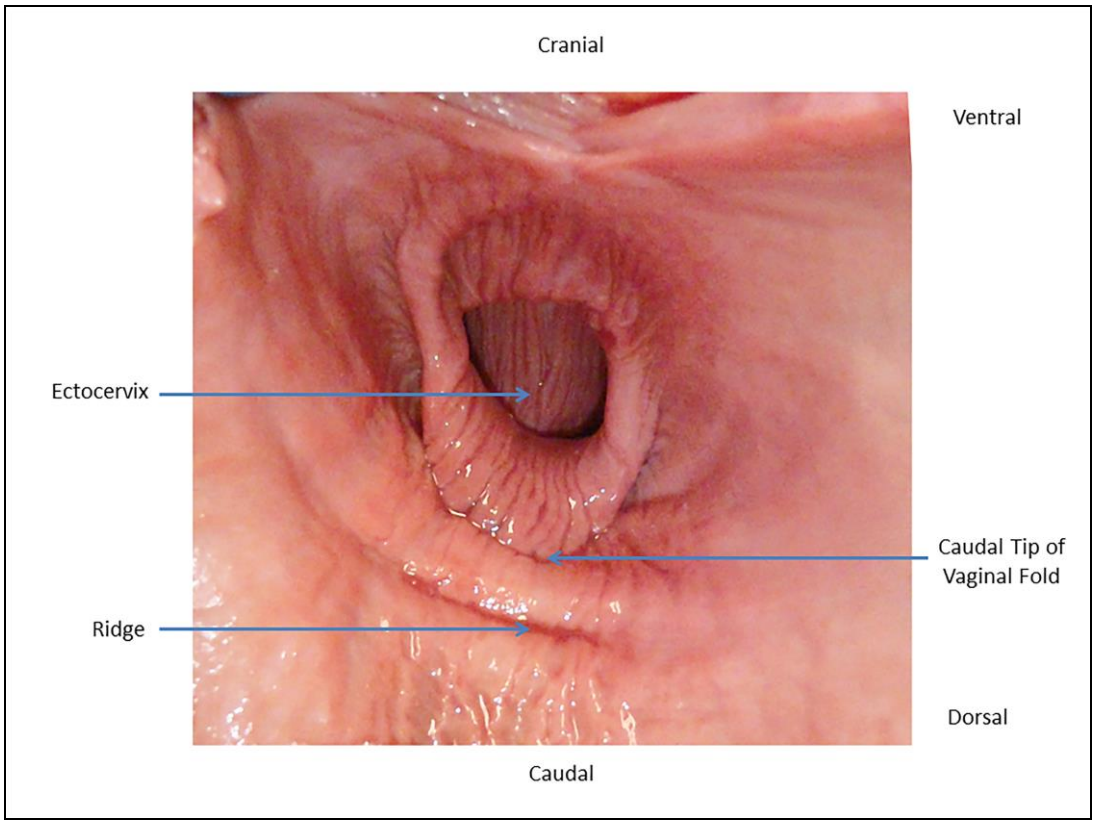

Figure 3-4. Dissected reproductive tract of a female common bottlenose dolphin in the transverse plane. The frozen-thawed specimen was oriented in dorsal recumbency. In this figure, the vaginal lumen was intact (we did not make a longitudinal incision). The vaginal fold encircled the entire interior circumference of the vagina and its distal tip projected caudally towards the vulva. The vaginal fold had the greatest length and created a recess on the dorsal side of the vaginal wall. The distal tips of the vaginal fold were serrated and uneven. A banded texture pattern was present along the longitudinal axis of the vaginal fold on the interior of the lumen. The ectocervix was visible through the lumen of the vaginal fold. A shallow transverse ridge was positioned caudal to the vaginal fold and did not extend along the complete circumference of the vaginal lumen.

The vagina was bisected in the transverse plane by a single vaginal fold, which was large, deep, and protruded from the entire width of the vagina caudally into the vaginal lumen (Figs. 3-1, 3-3, 3-4). One lactating sexually mature female from Florida and the one immature female from Virginia had a secondary vaginal fold that was shorter in length. In the mature specimen, the secondary vaginal fold was in the caudal vagina (caudal to the primary vaginal fold) and was substantially less developed than the primary vaginal fold (6 mm vs 29 mm in vaginal fold length, respectively). In the immature specimen, the secondary vaginal fold was in the cranial vagina and was moderately less developed than the primary vaginal fold (6 mm vs 10 mm in vaginal fold length, respectively). Only the primary vaginal fold data were included in the analysis. Across all specimens, the vaginal folds had the greatest lengths on the dorsal wall of the vagina (Fig. 3-4). The folds had fine textured bands running longitudinally on their interior surface (oriented towards the vaginal lumen; Figs. 3-1, 3-3, 3-4). The distal tips of the folds ranged from thin and serrated to thick and non-serrated (Figs. 3-1, 3-3, 3-4). The caudal vagina had up to two shallow transverse ridges (<5 mm in length). If these ridges were present, they were located in the cranial end of the caudal vagina, were often branching, did not extend around the entire circumference of the vaginal lumen, and were most prominent on the dorsal vaginal wall (Figs. 3-3, 3-4). No vaginal band (hymen) was observed in any specimen. The clitoris was keeled, dense, and projected into the genital slit. The glans (distal tip) of the clitoris pointed directly to the cranial limit of the vulva, located caudo-dorsally and demarcated by a darker tissue color than the interior of the vagina.

The raw reproductive tract measurements are presented in Table 3-2. The data scaled by body length are presented in Figures 3-5 and 3-6. We present data on all mature females for the sexual maturity state and geographic area analyses, regardless of reproductive state; this yielded a larger sample size and no differences were found between all mature specimens and resting-only specimens for any of the morphological measurements.

Sexual Maturity State

The left and right uterine horns were proportionally longer in sexually mature than immature dolphins, and much of the skew was driven by pregnant females (Fig. 3-5A, Table 3-2). The vaginal fold was marginally more developed (longer and therefore greater surface area) in sexually mature animals (Fig. 3-6A, Table 3-2).

Reproductive State

The upper reproductive tracts of pregnant females were greater in all measurements, except for the total reproductive tract length, compared to lactating or resting females (Fig. 3-5B, Table 3-2). There were no differences between lactating and resting females in any of the six measurements for the upper reproductive tract. One pregnant female had longer vaginal length measurements (total, cranial, and caudal) than lactating or resting females (Fig. 3-6B, Table 3-2). The other two pregnant females were incomplete specimens and their vaginal lengths could not be measured. Vaginal folds were wider in pregnant females than lactating females (Fig. 3-6B, Table 3-2). There was an outlier in

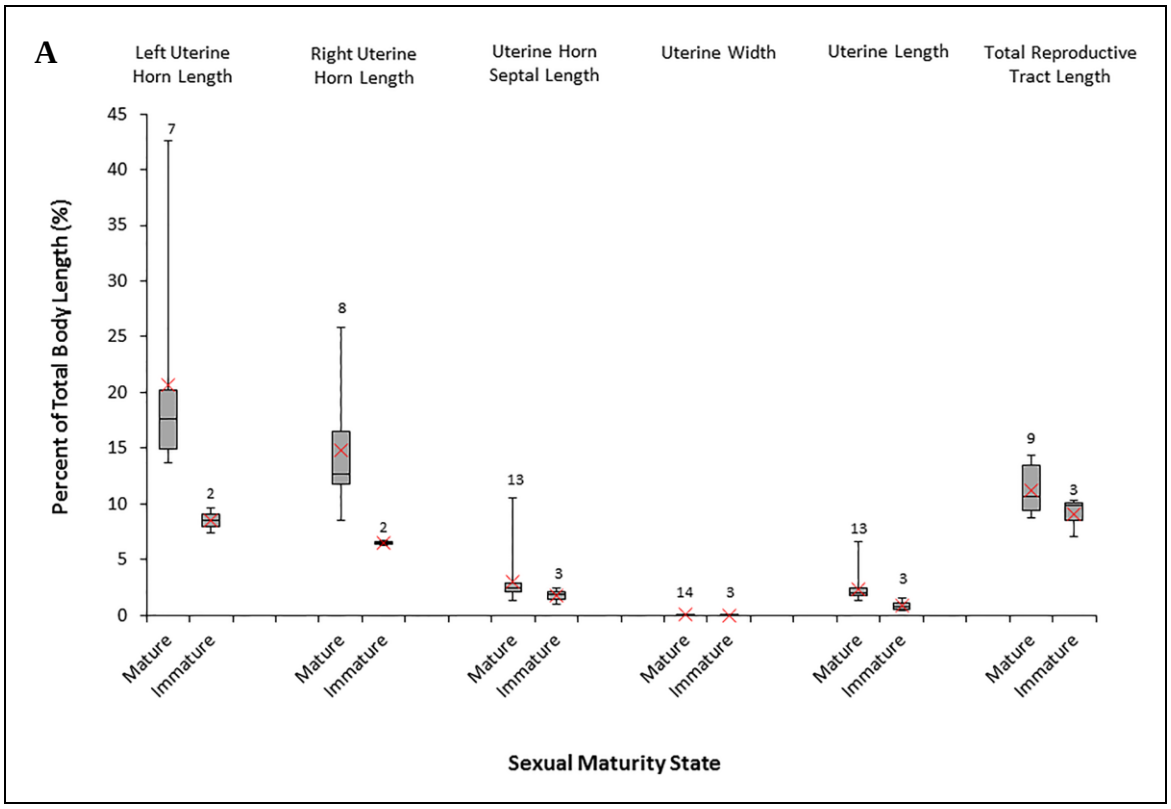

Figure 3-5. Tukey's box and whisker plots of the upper reproductive tract measurements of female common bottlenose dolphins. Specimens are divided by: (A) sexual maturity state (including all reproductive states), (B) reproductive state (adults only), and (C) geographic area (adults only). All data are shown as a percentage of the dolphin's total body length. The '-' represents the median. The red 'X' symbol denotes mean percentages. The specimen count for each measurement is above the whiskers.

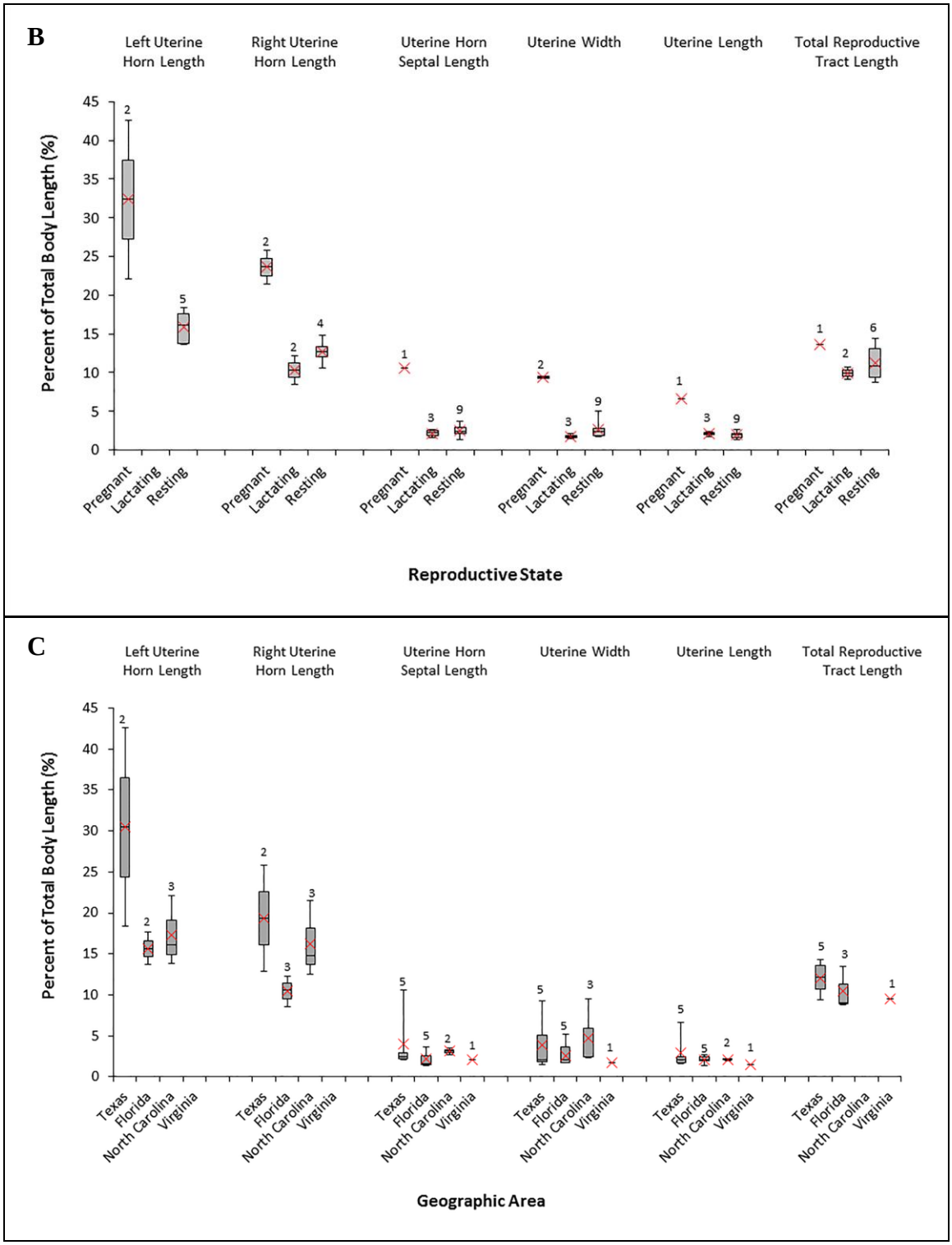

Figure 3-5, Continued.

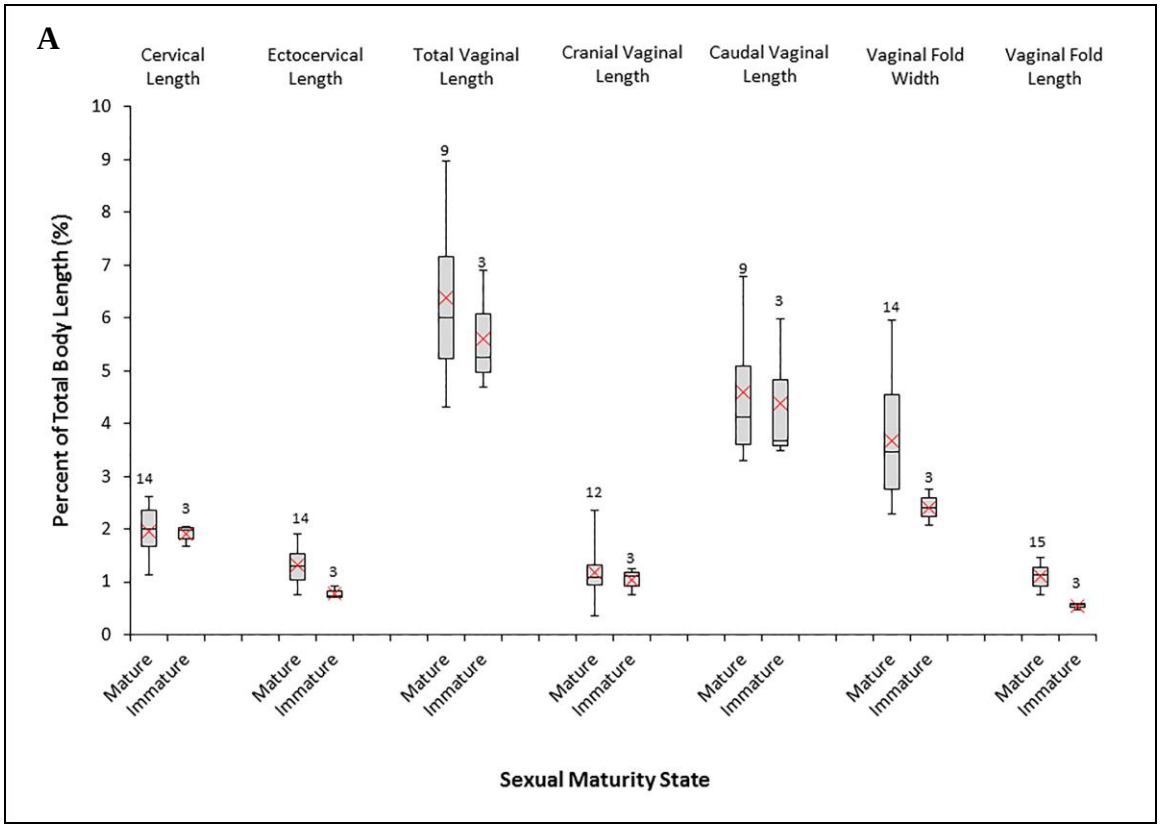

Figure 3-6. Tukey's box and whisker plots of the lower reproductive tract measurements of female common bottlenose dolphins. Specimens are divided by: (A) sexual maturity state (including all reproductive states), (B) reproductive state (adults only), and (C) geographic area (adults only). All data are shown as a percentage of the dolphin's total body length. The '-' represents the median. The red 'X' symbol denotes mean percentages. The specimen count for each measurement is above the whiskers.

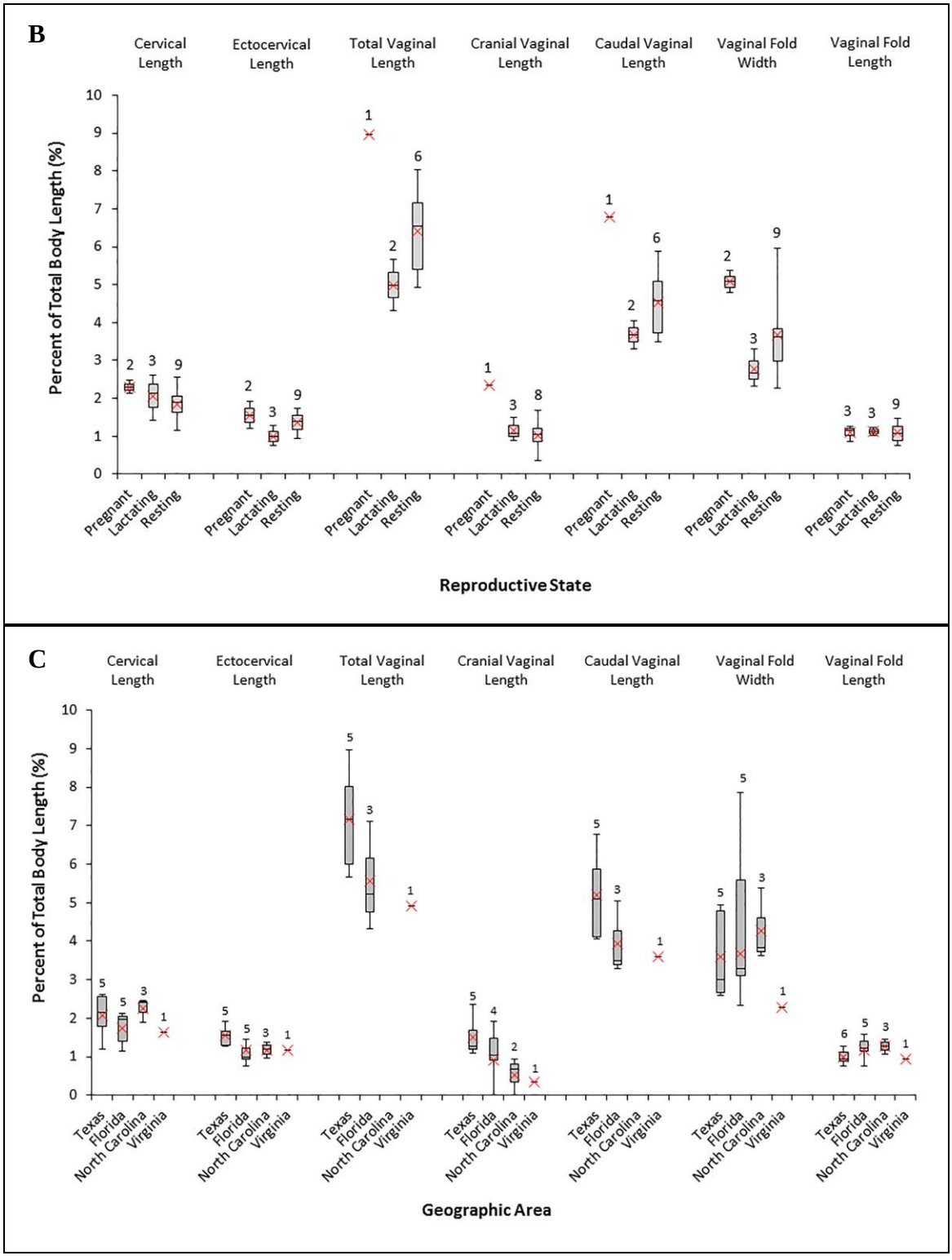

Figure 3-6, Continued.

the vaginal fold width data for resting females, and it is unclear if pregnant females also had wider vaginal folds than resting females.

Geographic Area

Adult specimens from the four geographic areas had overlapping ranges of body lengths (Table 3-2). Samples from the Texas population of dolphins had marginally longer left and right uterine horn lengths than samples from the Florida population (Fig. 3-5C, Table 3-2). The samples from the North Carolina population had slightly longer cervical lengths, vaginal fold widths, and vaginal fold lengths than those from Virginia (Fig. 3-6C, Table 3-2). All vaginal length measurements decreased gradually from Texas to Virginia (Fig. 3-6C, Table 3-2).

Histology

We assessed the microanatomy of five specimens, all of which had only one vaginal fold (Table 3-1). The microstructure of the cranial vaginal fold and caudal vaginal wall tissues consisted of smooth muscle bands (fascicles), mixed with dense irregular connective tissue, blood vessels, and adipose tissue (Fig. 3-7). Skeletal muscle was not found in any of the 100 micrographs analyzed. The data from 90 micrographs were included in the mixed model analysis. The variance partition coefficient indicated that 45% of the model variance was explained by inter-animal differences. There was no significant difference in estimated marginal means for the density of muscle banding between vaginal fold (N = 42, $\bar{x}$ = 49.29 ± 5.3%) and vaginal wall tissues (N = 48, $\bar{x}$ =

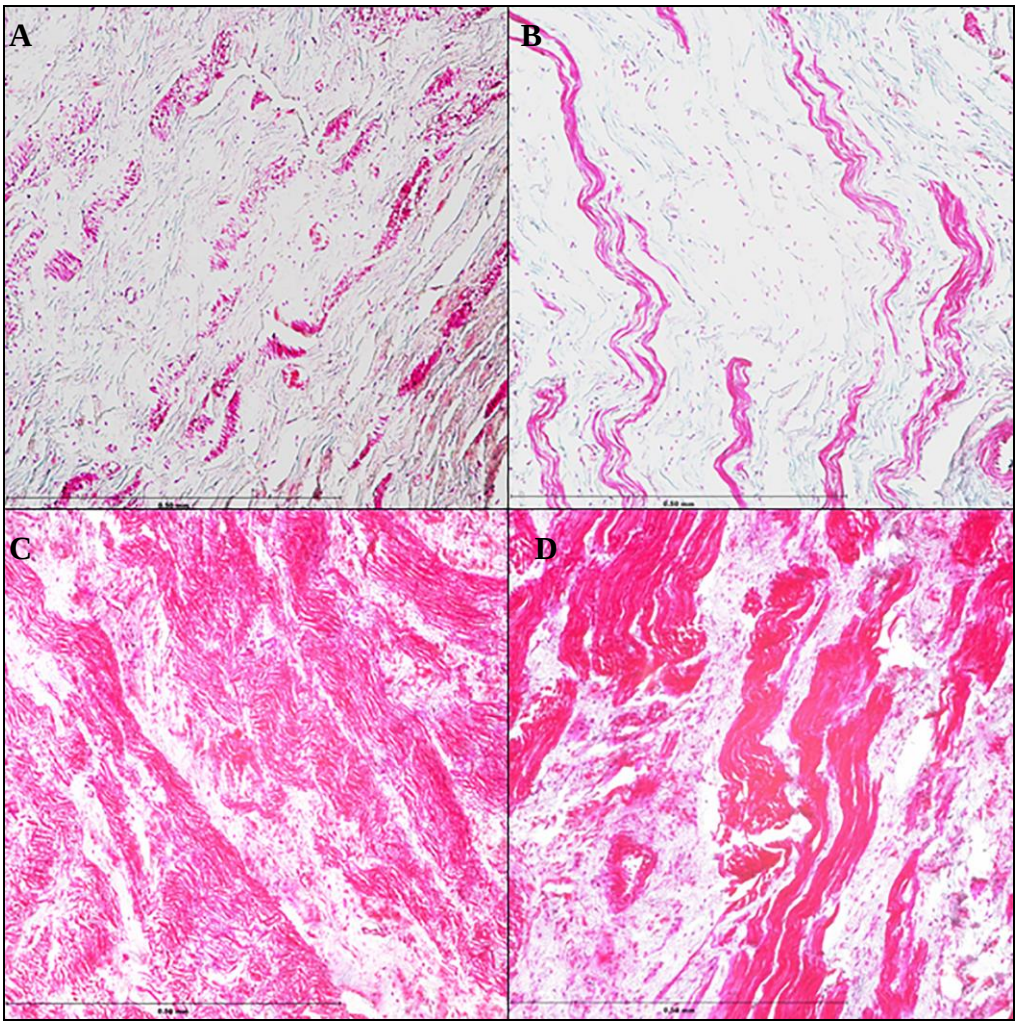

Figure 3-7. Micrographs of common bottlenose dolphin vaginal tissue at 20x magnification. The slides were stained with a modified Masson's trichrome stain to distinguish the muscle fascicles (pink) from connective tissue (green-blue), and from cell nuclei (black). The left micrographs (A and C) show vaginal fold tissue, while the right micrographs (B and D) show vaginal tissue collected from the caudal vagina. The top two micrographs (A and B) show low densities of muscle banding from the same adult dolphin, while the bottom two micrographs (C and D) show high densities of muscle banding from a second adult dolphin.

44.56 ± 5.3; z = -1.84, p= 0.07; Fig. 3-7) or between sexually mature (N = 57, $\bar{x}$ = 45.47 ± 6.6) and immature specimen tissues (N = 33, $\bar{x}$ = 49.01 ± 8.1; z = 0.34, p = 0.74). However, there was a significant interaction between tissue type and maturity state (P = 0.002), with the significant differences (z = -3.51, p = 0.003) limited to the vaginal walls ($\bar{x}$ = 41.92 ± 8.3) and folds ($\bar{x}$ = 57.1 ± 8.5) of sexually immature specimens.

Discussion

The goal of our study was to describe the anatomy of female common bottlenose dolphin reproductive tracts and to lay the foundation for future investigation of potential functions of vaginal folds. We found little variability in the morphological measurements of reproductive tracts between sexually mature and immature specimens and across reproductive states. Female common bottlenose dolphins generally had only one large vaginal fold. We did not find skeletal muscle or differences in the densities of smooth muscle bands between the cranial vaginal folds and caudal vaginal walls or between states of sexual maturity.

Female Anatomy: Landmarks

Our observations of more *corpora lutea* and/or *corpora albicantia* on the left than right ovary and of longer left than right uterine horns are consistent with what is known for common bottlenose dolphins (Robeck et al., 2005) and some other odontocetes (toothed whales) after their first pregnancy (Ohsumi, 1964; Sljper, 1966). *Corpora lutea* and *corpora albicantia* accumulate on ovaries following ovulations and pregnancies

(Mackintosh and Wheeler, 1929; Ohsumi, 1964; Sljper, 1966; Harrison, 1969; Harrison and Ridgway, 1971; Plön and Bernard, 2007; Rommel et al., 2007). A fetus had been removed from the left uterine horn of all three pregnant specimens that we dissected. Our data support observed laterality in ovulation and pregnancy in common bottlenose dolphins, and are congruent with previous reports of odontocetes' tendency towards left uterine horn ovulation, implantation, and development (Slijper, 1966; Robeck et al., 2005). Both the left and right uterine horn lengths that we report for common bottlenose dolphins were substantially longer than those reported by Robeck et al. (1994). This disparity may result from our inclusion of the oviduct in the left and right uterine horn length measurements. Our findings suggest that the presence or absence of fine longitudinal bands in the uterine horns may be useful in categorizing sexual maturity states if the ovaries are not present. The cetacean uterus is bicornate and is completely separated from the lower reproductive tract by the cervix (Sljper, 1966). The uteri in the current study were shorter in length (scaled by body length), yet similar in appearance to the bicornate uteri of dogs, pigs, and elephants that develop the fetus within the uterus and not the uterine horn (Schroeder, 1990). Our measurements of uterine length are congruent with those reported by Robeck et al. (1994) for common bottlenose dolphins.

The cervix was almost the same length as the uterus in both this and Robeck et al.'s (1994) study of common bottlenose dolphins. The uterus length relative to the cervix length is highly variable across mammals (König and Liebich, 2007). The presence of longitudinal bands in the cervix could provide a passageway for spermatozoa to reach the uterus by travelling between the bands and avoiding the dense

mucus barrier produced by females in the central cervical canal (bovines, Mullins and Saacke, 1989; goats and cattle, Mattner, 1968). The presence of similar longitudinal bands on the interior surface (lumen-oriented) of the vaginal folds (Pycraft, 1932; Harrison, 1949) supports the hypothesis that vaginal folds function in sperm movement. Our observed patterns of the greatest ectocervical and vaginal fold lengths on the dorsal wall of the vagina have been reported in other cetaceans (bowhead whales, *Balaena mysticetus*; Tarpley and Hillmann, 1999), although the functional importance is unclear.

The majority of our measurements focused on the lower reproductive tract, and specifically the vaginal folding. Although the common bottlenose dolphins in Robeck et al.'s (1994) study had longer body lengths than those in our study, the total vaginal lengths of our specimens were 2 cm longer on average. In contrast to other cetaceans, common bottlenose dolphins have one large vaginal fold (Green, 1977). However, one sexually mature and one sexually immature specimen in our study possessed a second, smaller vaginal fold. The second vaginal fold varied in its degree of development, position relative to the primary fold (cranial or caudal), and shape of its distal tip. There was individual variation in vaginal morphology, although most characteristics were shared among the specimens we observed. In short-beaked common dolphins (*Delphinus delphis*) and other unspecified cetacean species, vaginal structures decreased in length and width from the cranial to the caudal direction (Hunter, 1787; Pycraft, 1932). However, the opposite pattern was found in bowhead whales (Tarpley and Hillmann, 1999).

Variation Across Sexual Maturity State, Reproductive State, and Geographic Area

We found a small range of variation among specimens in the general reproductive tract morphology of female common bottlenose dolphins. Our finding expands the pool of suitable specimens for future research; we suggest that specimens, regardless of their sexual maturity state, reproductive state, or geographic location, can be used interchangeably in comparative morphological studies of the cetacean vagina.

Maturation-related developmental shifts occur in the morphology of some tissues associated with reproduction in mammals (e.g., genital swelling in primates, Nunn, 1999). However, the only variation observed in this study between sexually mature and immature specimens in the lower reproductive tract was the extent of vaginal fold development, and the difference was minimal. In the upper reproductive tract, sexually mature specimens were observed to have relatively longer left and right uterine horn lengths than immature specimens, as expected, just as humans display maturity-related increases in uterine size (Salardi et al., 1985; Herter et al., 2002). Based on our observations of minimal variation in reproductive tract morphometrics, sexually immature specimens look like small mature specimens.

As expected, pregnant females had more distended upper reproductive tracts than lactating and resting sexually mature dolphins to accommodate the developing fetus, amniotic cavity, and placenta (Wislocki and Enders, 1941). Changes in uterine dimensions reflect vascular alterations of the mucosa and are related to reproductive state in other cetaceans as well (fin whales, *Balaenoptera physalus,* Mackintosh and Wheeler, 1929). Lower reproductive tract variations were minimal across reproductive

states. We cannot comment on relative vaginal length variations since we were only able to obtain measurements for one of the three pregnant females. The few variations in the lower reproductive tract morphometrics of pregnant and non-pregnant females could reflect individual variation or adaptations related to conception, pregnancy, or estrus, as seen in primates (Nunn, 1999).

Specimens from Texas, Florida, North Carolina, and Virginia represent different populations of common bottlenose dolphins (Rosel et al., 2011). There were few clear patterns of variation in upper or lower reproductive tract morphology across the population samples we assessed. Albeit tentative, the differences we observed could potentially indicate real geographic variation across populations of common bottlenose dolphins (e.g., decreases in vaginal lengths from Texas to Virginia). As *T. truncatus* has a near-global distribution, investigations of reproductive morphology at a broader geographical scale and with a larger sample size are warranted and could yield interesting comparisons.

Histology

No skeletal muscle was present in the cranial or caudal vaginal wall of common bottlenose dolphins, congruent with other mammals (domestic cats, *Felis catus*; Rosengren and Sjöuber, 1967; domestic rabbits, *Oryctolagus cuniculus*; Rodríguez-Antolín et al., 2009; rats, *Rattus norvegicus*; Berger et al., 2005). No changes were found in the density of muscle banding between cranial vaginal fold and caudal vaginal wall tissues in common bottlenose dolphins, in contrast to Harrison's (1949) results for

long-finned pilot whales. He qualitatively described muscles in the cranial vaginal fold tissue as large and sparse and muscles in the caudal vaginal wall tissue as small and abundant. Harrison (1949) did not differentiate between the microstructure of fetal and adult vaginal fold and wall tissues, potentially indicating no maturity-specific variation. However, we found that the variance observed in percent muscle banding between tissue types was significantly explained by differences within sexually immature specimens. We emphasize the importance of assessing ontogeny to understand functionality.

Form and Function

A number of non-mutually exclusive hypotheses – sexual selection, natural selection, and phylogenetic similarity and constraint, among others – might explain the presence of cetacean vaginal folds (see above; Clarke et al., 1994). It has been hypothesized that vaginal folds might function in selective sperm movement (Clarke et al., 1994). We found no evidence that the common bottlenose dolphin vaginal wall muscle is under somatic control. However, in other taxonomic groups where females eject sperm, muscle contractions also appear to be under autonomic control (dunnocks, *Prunella modulari*; Davies, 1983; damselflies, *Calopteryx haemorrhoidalis asturica*; Cordoba-Aguilar, 1999). The stimulus itself may be more important than the muscle type in inducing spontaneous or reflexive contractions (coital reflex, Carro-Juarez and Rodríguez-Manzo, 2000; acoustic reflex, Thompson et al., 1980). Further analyses of the muscle architecture of dolphin vaginal walls, particularly focusing on the innervation,

histochemistry, biophysical properties, and force production, will advance the understanding of vaginal fold functionality (bottlenose dolphin muscles, Pabst, 1993; Etnier et al., 2004). On the one hand, vaginal folds could divert sperm away from the cervix and upper reproductive tract; vaginal folds project caudally into the lumen of the vagina and form deep dead-end crypts. Semen trapped caudal to the vaginal folds could come into contact with lethal seawater. On the other hand, the longitudinal textured bands on the interior of the vaginal folds could provide a passageway for spermatozoa to reach the upper reproductive tract, and/or the vaginal folds could hold or reduce the loss of semen. For example, Green (1977) reported more fluid that looked like semen cranial than caudal to the vaginal fold in a dissected female common bottlenose dolphin. Future studies that tag and track the movements of semen through the female reproductive tract *in vivo* will be able to test if vaginal folds create storage crypts (fruit flies, *Drosophila melanogaster*; Manier et al., 2010) or facilitate semen uptake or expulsion.

Much of a female's reproductive success relates to her reproductive anatomy, yet research on female genitalia has been largely under-represented in the scientific literature (Ah-King et al., 2014). In cetaceans, female mating tactics have been generally overlooked, often because of logistical challenges. Our exploration of the reproductive tract morphology of the female common bottlenose dolphin establishes the framework necessary to conduct intra- and interspecific comparative studies; we can now begin to test alternative hypotheses for the evolution of these unusual vaginal folds. We

demonstrate that sexual maturity state, reproductive state, and geographic area do not appear to influence vaginal morphology within *T. truncatus* from the southeastern USA. While mating tactics have been inferred from female reproductive tract morphology in eutherian mammals (Gomendio and Roldan, 2003), few other studies have recognized the potential for vaginal morphology to advance our understanding of mammalian mating systems.

Chapter III Supplement[2]

Diagnostic imaging techniques have been of great utility to live cetacean reproductive biology, such as captive breeding programs (e.g., Robeck et al., 1994, 2010; Brook, 1997). The ability to visualize vaginal structures *in situ* may provide details unavailable when reproductive tracts are dissected, and thus help determine relationships between form and function. In addition to anatomical topography, different densities of soft tissues can also be explored in cetaceans with computed tomography (CT) scans (e.g., Mckenna et al., 2007). To my knowledge, CT scans have not been conducted on the reproductive tracts of cetaceans. My objective was to assess if CT scans are comparable to dissections for measuring landmarks.

Materials and Methods

Double-contrast vaginograms were performed on three reproductive tracts of common bottlenose dolphins (*Tursiops truncatus*) obtained in Texas. One sexually mature (resting) specimen used in the gross morphological analysis in chapter III and two additional sexually immature specimens not used in the gross morphological analysis were transferred intact to Texas A&M University's Veterinary Medical Teaching Hospital in College Station for CT scans. CT scans were performed prior to dissections.

[2] CT scan were performed in collaboration with Corey Wall and Hollye Callis

The mature specimen was completely intact while the two immature specimens were missing tissue cranial to the cervix.

The specimens were placed in a dorsal recumbency with a cranial orientation to the gantry. A 28 French Foley catheter (9.33 mm diameter) was inserted into the vaginal cavity. The catheter was positioned caudal to the vaginal folds. The balloon of the Foley catheter was filled with 30 ml of ambient air to distend the vagina. To obtain an airtight seal of the vaginal cavity, the external vaginal opening was sutured closed around the catheter using a simple continuous suture pattern, which was then over-sewn by a modified purse string suture pattern with # 2 Ethilon (Ethicon). A seal was made in the uterus as far cranial to the cervix as possible. The uterus was sealed in the sexually mature specimen by performing a circumferential ligature with 1/8" umbilical tape. The two sexually immature specimens had insufficient amounts of uterine tissue cranial to the vagina for a circumferential ligature and were sealed with a curved carmalt surgical clamp.

A total of 10 ml of an iodinated intravenous contrast (Conray 400 iothalamate sodium injection usp 66.8%; 400 mg/mL organically bound iodine) was administered into the vaginal cavity via the Foley catheter. The vaginal cavity was then distended with air introduced by hand via a 60 cc catheter syringe. Distention of the vaginal cavity was assessed visually and by the amount of tactile pressure on the plunger of the catheter syringe. Contiguous transverse images of the specimens were obtained in a cranial to caudal direction using a high resolution, multi-detector, 40 slice helical scanner (Siemens Somatom Definition A5). Slice thickness was set at 0.6 mm. The images were

reconstructed in the transverse, sagittal, and horizontal planes in both bone and soft tissue algorithms. Following the scanning, the reproductive tracts were drained of iodine and flushed with water for subsequent dissections.

The evaluation of the vaginal cavities was performed with eFilm viewing software (Merge Healthcare, v.3.4.0.10). All measurements were acquired using the linear measurement function. Images were evaluated on the bone reconstructed algorithm at a window width of 4,000 and window level of 700. Each measurement was initially subjectively assessed in all 3-dimensions. The plane that best showed the measurement of interest was then selected for the comparative measurement. Only measurements of the lower reproductive tract were analyzed (Fig. 3-1B), since the specimens were sealed at the uterus. The cranial limit of the vulva landmark (Fig. 3-1A) was not visible on the CT scans. Measurements of the lower reproductive tract are detailed in chapter III and included the cervical length, ectocervical length, cervical thickness (cranial-caudal thickness of the ectocervix at its thickest point; calipers were positioned on the cranial and caudal side of the ectocervix), cranial vaginal length (from the base of the ectocervix and from the caudal tip of the ectocervix), vaginal fold width, vaginal fold length, vaginal fold thickness (cranial-caudal thickness of the vaginal fold at its thickest point; calipers were positioned on the cranial and caudal side of the vaginal fold), and number of vaginal folds. The percent differences between the direct and CT scan measurements were calculated consistently using the direct measurements as the denominator.

Results

Variable numbers of the nine potential measurements could be collected by both direct measurements and CT scan methods for each specimen. All 9 measurements were obtained for the single sexually mature specimen (specimen 1), whereas only 8 and 6 measurements were collected for the two sexually immature specimens (specimens 2 and 3, respectively; Table 3-3). The percent differences between CT scan and direct measurements averaged 66.6%. CT scan measurements were greater than direct measurements in 16 of the 23 collected data values (Table 3-3). There were no clear patterns of consistency in the differences between direct and CT scan measurements.

Discussion

CT scans were not comparable to direct measurements during dissections for measuring vaginal landmarks in common bottlenose dolphins. Measurements obtained from CT scans yielded consistently different results from dissections of the same specimens using the same anatomical landmarks. Additionally, there was inconsistency with which technique yielded higher values. There are several possibilities why the two techniques yielded such varying results. There were limitations associated with using each technique. Some anatomical landmarks were not clearly visible on the CT scans. For example, the change in longitudinal banding pattern used to delineate the cranial end of vaginal folds during dissections was not visible in the CT scans. Challenges physically manipulating the reproductive tracts could also lead to over- or under-estimates of measurements. For example, it was

Table 3-3. Comparison of direct and CT scan measurements. Mean (± SD) measurements collected from the reproductive tracts of up to three female common bottlenose dolphins using calipers during dissections or CT scans. All length, width, and thickness measurements are in mm. Values are raw data and are not scaled by body length. Any negative percent difference indicates that the direct measurement exceeded the CT scan measurement.

	Specimen 1 (Sexually Mature)			**Specimen 2** (Sexually Immature)			**Specimen 3** (Sexually Immature)		
	Direct	CT	% Difference	Direct	CT	% Difference	Direct	CT	% Difference
Cervical Length	3	30.66	57.60	N/A	N/A	N/A	N/A	N/A	N/A
Ectocervical Length	5	33	24.24	10	12.7	21.26	8	13.42	40.39
Cervical Thickness	7	3.11	-446.62	7	3.15	-122.22	N/A	N/A	N/A
Cranial Vaginal Length-From Cervix Base	8	32.01	-18.71	5	11.92	58.05	12	12.55	4.38
Cranial Vaginal Length-From Cervix Distal Tip	5	7.69	-95.06	5	3.44	-45.35	N/A	N/A	N/A
Vaginal Fold Width	7	125.48	62.54	24	38.38	37.47	19	50.42	62.32
Vaginal Fold Length	6	32	18.75	2	5.78	65.40	7	2	250
Vaginal Fold Thickness	0	6.47	-54.56	4	6.98	42.69	7	7.29	3.98
Number of Folds	1	1	0	1	1	0	1	1	0
Average % Difference			86.5			49.1			60.2

difficult to reach the base of the ectocervices and folds using the calipers or scales. Similarly, the linear measurement tool used to analyze the CT scans was not robust to the curved distal tips of the ectocervices and vaginal folds. Variation in the quality of the specimens also likely contributed to inconsistent data trends within and between techniques. For example, flaccid tissue is more likely to blend in with the mucosa and remain undetected by the scan. The rigidity of the tissue can also correspond with the state of decomposition (Janaway et al., 2009). Since the two sexually immature specimens had minimal uterine tissue available to create the tight seal, the cervical tissue may have been compressed during the CT scans. Another potential source of error was variation in the orientation of the specimens to the CT scanner. Although external landmarks were aligned accurately on the scanner table, the internal cervical region was angled slightly out of the sagittal plane in the two sexually immature specimens. Furthermore, distension of the reproductive tracts with contrast agents can change the dimensions of internal structures, and these changes may vary with pressure or tissue quality. Much of the variability in our pilot CT scan study could be reduced in the future with additional experience and with exclusive use of high quality (e.g., less than 24 hours post-mortem, not frozen-thawed) and completely intact reproductive tracts.

CHAPTER IV

PATTERNS OF CETACEAN VAGINAL FOLDS YIELD INSIGHTS INTO FUNCTIONALITY*

Summary

Female cetaceans possess unusual and complex foldings of the vaginal wall, of unknown function(s). These folds project into the lumen of the vagina and can occupy much of the total vaginal space. The patterns of vaginal length and cumulative vaginal fold length were assessed with body length and with each other to derive insights into functionality. The reproductive tracts of 58 female cetaceans (19 species, 5 families) were dissected. Phylogenetically controlled regressions were used to test the hypotheses that mean total body length was a predictor of mean vaginal length and mean cumulative vaginal fold length, and that these two vaginal traits were associated with each other. Body length positively and significantly predicted vaginal length and cumulative vaginal fold length, but vaginal length and cumulative vaginal fold length themselves were not significantly correlated. The data indicate that non-scaling selection pressures may also account for the variability observed in vaginal morphology among cetaceans. Natural and sexual selection functional hypotheses are highlighted. Vaginal folds may present physical barriers that obscure the pathway of seawater and/or sperm travelling through the vagina.

*This chapter is intended for publication in collaboration with Christopher Marshall, Sarah Mesnick, and Bernd Würsig.

Introduction

Whales, dolphins, and porpoises demonstrate extensive morphological, physiological, and behavioral adaptations that enable them to thrive in the aquatic environment (reviewed in Howell, 1930; Kellogg, 1938; Pabst et al., 1999; Reidenberg and Laitman, 2007; Gatesy et al., 2013; Berta et al., 2015). Adaptations of the reproductive system include internal location of the testes within the abdominal cavity (enhanced streamlining; Slijper, 1962; De Smet, 1977) and a modified vascular network, or *rete mirabile*, of the testes and uterus for thermoregulation (Rommel et al., 1992; Rommel et al., 1993; Pabst et al., 1998). The female genitalia of cetaceans have received few functional investigations, just as female genitalia in many taxa remain unstudied (Ah-King et al., 2014).

Female cetaceans possess unusual folds of tissue in their vaginas, which are of unknown function (reviewed in Clarke et al., 1994; Orbach et al., 2016/chapter III). Vaginal folds are transverse protrusions of the vaginal wall into the vaginal lumen, with the distal tips often oriented caudally (Pycraft, 1932). There is a broad diversity in vaginal fold morphology across cetaceans (Ommanney, 1932). In many species, these vaginal folds are located in the cranial end of the vagina (Schroeder, 1990) and typically decrease in size cranially-to-caudally (Hunter, 1787; Pycraft, 1932; but see Tarpley and Hillmann, 1999). Similar vaginal structures are present in hippopotamuses (e.g., *Hippopotamus amphibius*; Laws and Clough, 1966), which are the closest terrestrial relatives to cetaceans and also mate in the water. Vaginal folds appear to be unique to cetaceans and their closest relatives, the artiodactyls; similar structures have been

reported as absent in non-cetacean marine mammals (phocids; Harrison et al., 1952; Australian sea lion, *Neophoca cinerea*; Tedman, 1991; California sea lion, *Zalophus californianus*; Colegrove et al., 2009; Amazonian manatee, *Trichechus inunguis* Natterer; Rodrigues et al., 2008; sea otter, *Enhydra lutris*; Sinha et al., 1966).

Several non-mutually exclusive hypotheses have been proposed for the functions of cetacean vaginal folds, although none have been empirically tested. Vaginal folds have most often been suggested to function as adaptations for copulation in the marine environment. Seawater is lethal to common bottlenose dolphin sperm (*Tursiops truncatus*, Schroeder and Keller, 1989), and presumably to the sperm of all cetaceans that mate in marine environments. Thus, vaginal folds, in addition to the cervix, may function to prevent seawater from contacting the ejaculate when the penis is inserted or withdrawn (Slijper, 1962; Green, 1972, 1977; Chen et al., 1984; Schroeder, 1990; Robeck et al., 1994).

Vaginal folds might facilitate gestation and parturition. For example, vaginal folds have been hypothesized to counteract diving-related pressure changes and prevent the "expulsion of the fetus from the womb" (Kellogg, 1938). Vaginal folds might aid in parturition by distending the reproductive tract (Meek, 1918; Slijper, 1962).

Vaginal folds could also play a role in mating. For example, they may be adaptations to induce sperm competition (Clarke et al., 1994). Cetacean semen does not coagulate because males lack seminal vesicles and bulbourethral glands (Slijper, 1966; Harrison, 1969). Accordingly, vaginal folds might facilitate sperm retention and increase fertilization success. Vaginal folds might constitute physical barriers to prevent the loss

of semen (Meek, 1918; Harrison, 1969), or provide passageways for sperm transport along the fine longitudinal bands found on the vaginal folds (Orbach et al., 2016/ chapter III), as observed in the cervices of some terrestrial mammals (goats and bovines, Mattner, 1968; Mullins and Saacke, 1989). They may also physically stimulate the penis during copulation and promote ejaculation, and possibly propel semen towards the uterine horns by muscle contractions to facilitate fertilization (Meek, 1918; Harrison, 1969).

The influence of body size on evolution and adaptations is well recognized (e.g., Schmidt-Nielsen, 1984; LaBarbera, 1989; Biewener, 2015). Body size is one of the most basic but important adaptation to aquatic living in marine mammals (e.g., Pabst et al., 1999). An analysis of the relationships of cetacean vaginal morphology with body size, while controlling for phylogenetic effects, provides a necessary basis for assessing the diversity and functions of vaginal folds. This chapter examines the hypothesis that vaginal length and cumulative vaginal fold length scale proportionally with body length, and that vaginal length predicts cumulative vaginal fold length in cetaceans.

Materials and Methods

Data Collection

Specimens were opportunistically obtained from marine mammal stranding networks throughout the coastal USA and from New Zealand. Specimens were collected under National Marine Fisheries Service (NMFS) salvage permit letters and an institutional Convention on International Trade in Endangered Species of Wild Fauna and Flora

permit (CITES Mammal Import Permit: 15US774223/9; MMPA/ESA research permit: 14097). Entire reproductive tracts (external uro-genital slit to ovaries) were excised from fresh (< 24 hours post-mortem) or moderately decomposed deceased cetaceans. The reproductive tracts were frozen immediately and transferred to necropsy facilities located at Texas A&M University at Galveston or the National Oceanic and Atmospheric Administration's (NOAA) Southwest Fisheries Science Center. The marine mammal stranding networks provided data on the date and location of each stranding, sexual maturity state, and total body length. Ideally, all specimens from any given species would represent only one age class. Due to the opportunistic nature of specimen acquisition and because no adult samples were obtained for six of the species included in this study, the specimens used were sexually mature and immature females from a range of stranding locations (Table 4-1). Minimal variation in vaginal morphology measurements has been found between sexual maturity states within at least one species (*T. truncatus*; Orbach et al., 2016/chapter III).

The reproductive tracts were positioned in dorsal recumbency and bisected by a longitudinal midline incision, from the clitoris to the external bifurcation of the uterine horns. Two measurements were collected following Orbach et al. (2016/chapter III)-vaginal length and cumulative vaginal fold length. The vaginal length was a cranial-to-caudal straight line measurement from the base of the ectocervix (portion of the cervix in the vaginal lumen, where it meets the vaginal wall) to the cranial limit of the vulva (Fig. 4-1). The vaginal length was measured with calipers (+/- 0.02 mm) down the midline of the reproductive tract along the dorsal vaginal wall. The vaginal fold length was a

Table 4-1. Counts and measurements of the specimens included in regressions divided by species, state of sexual maturity, and geographic area. The specimen count is divided into sexual maturity state based on information provided by the marine mammal stranding networks. One mean value of the number of vaginal folds, vaginal fold length, and cumulative vaginal fold length is provided per species. Measurements were not scaled by body length. The U.S. state (or country for New Zealand) where each specimen stranded is listed.

Species	No. of Sexually Mature Specimens	No. of Sexually Immature Specimens	Mean No. Vaginal Folds	Mean (±SD) Vaginal Length (mm)	Mean (±SD) Cumulative Vaginal Fold Length (mm)	Stranding Location(s)
Family Balaenoptera						
Balaenoptera acutorostrata	0	1	5	241.4 ± 0	71.0 ± 0	Virginia
Family Delphinidae						
Delphinus capensis	2	0	1.5	117.0 ± 37.9	17.5 ± 0.7	California
Delphinus delphis	3	2	1.4	128.2 ± 18.6	17.8 ± 6.5	Massachusetts, North Carolina
Globicephala macrorhynchus	1	0	4	209.0 ± 0	64.0 ± 0	Florida
Globicephala melas	0	1	4	180.8 ± 0	12.2 ± 0	Massachusetts
Lagenorhynchus acutus	1	0	5	184.5 ± 0	37.0 ± 0	Massachusetts
Lagenorhynchus albirostris	1	0	7	209.0 ± 0	51.0 ± 0	Massachusetts
Lagenorhynchus obliquidens	1	3	6.5	157.8 ± 47.3	55.0 ± 14.0	California, Oregon
Lagenorhynchus obscurus	3	0	3.3	77.6 ± 3.9	24.0 ± 2.6	New Zealand

Table 4-1, Continued.

Species	No. of Sexually Mature Specimens	No. of Sexually Immature Specimens	Mean No. Vaginal Folds	Mean (±SD) Vaginal Length (mm)	Mean (±SD) Cumulative Vaginal Fold Length (mm)	Stranding Location(s)
Orcinus orca	0	2	5	278.8 ± 150.5	34.0 ± 21.2	Alaska, New Zealand
Stenella frontalis	0	1	2	99.0 ± 0	48.0 ± 0	North Carolina
Tursiops truncatus	9	3	1.5	138.1 ± 42.7	25.4 ± 9.2	Florida, Texas, Virginia
Family Kogiidae						
Kogia breviceps	2	0	5.5	765.0 ± 75.0	219.5 ± 119.5	Florida, North Carolina
Kogia sima	0	1	2	375.0 ± 0	32.0 ± 0	Florida
Family Phocoenidae						
Phocoena phocoena	12	4	6.5	194.2± 61.3	80.7± 36.3	Alaska, California, Massachusetts, Oregon, Washington
Family Ziphiidae						
Mesoplodon bidens	1	0	2	662.0 ± 0	53.0 ± 0	Massachusetts
Mesoplodon europeaus	1	1	3	236.6 ± 12.9	21.0 ± 7.1	Florida, North Carolina
Mesoplodon peruvianus	0	1	1	243.8 ± 0	10.0 ± 0	California
Mesoplodon stejnegeri	1	0	3	617.0 ± 0	70.0 ± 0	Oregon

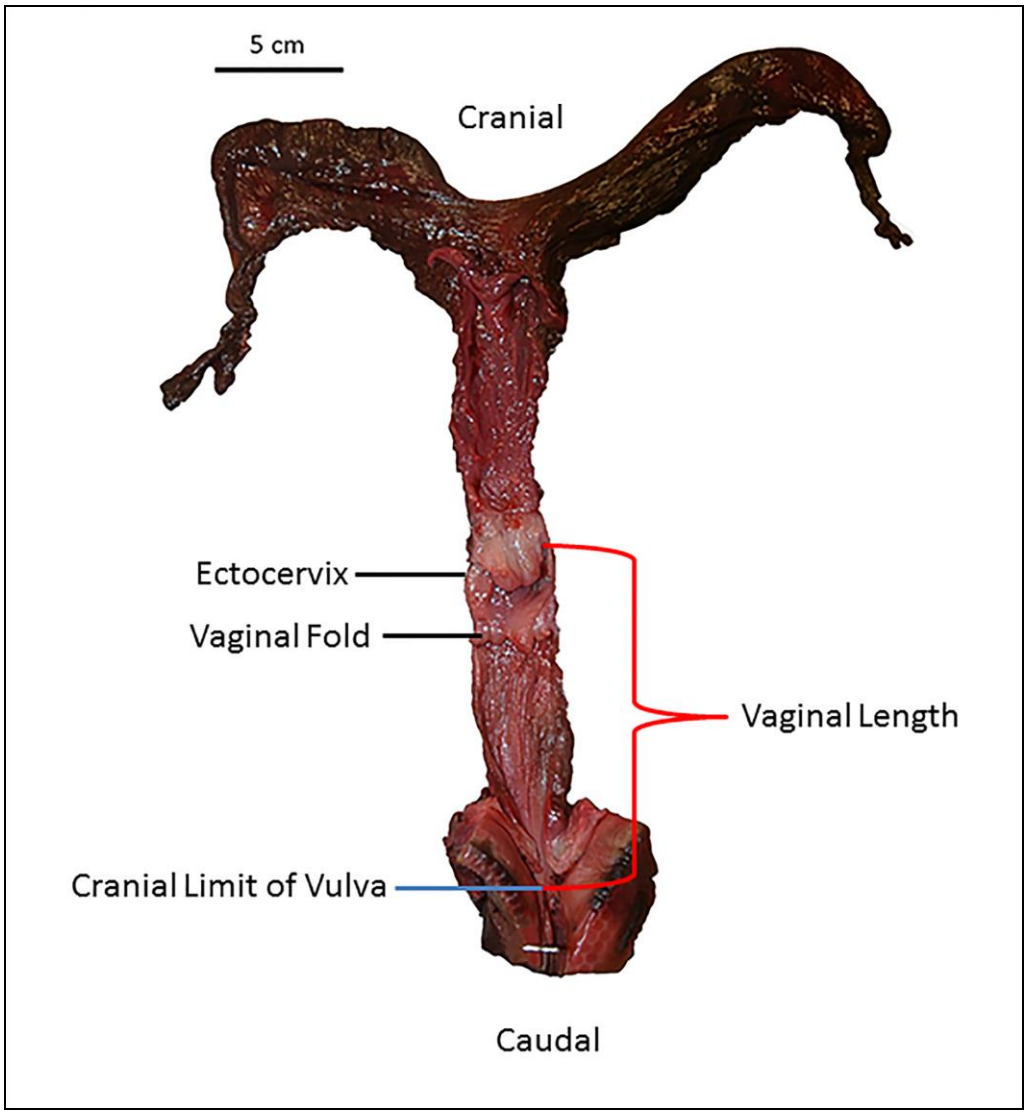

Figure 4-1. Vaginal length measurement of an adult female short-beaked common dolphin (*Delphinus delphis*). The reproductive tract is oriented in dorsal recumbency and splayed open. Vaginal length was measured with calipers down the midline of the dorsal vaginal wall. The measurement was taken in cranial to caudal orientation from the base of the ectocervix to the cranial limit of the vulva.

straight line measurement from the base of the vaginal fold (where it met the vaginal wall) to its distal tip that projected into the lumen of the vagina (Fig. 4-2). The vaginal fold length was measured with a scale positioned on the dorsal side of the vaginal fold. The cumulative vaginal fold lengths were summed for each specimen. A vaginal fold was defined as any folding of the vaginal wall 90^{o} to the long axis of the body and at least 0.5 mm in length. Any vaginal fold less than 0.5 mm in length was rounded to 1 mm. Total body lengths were provided by marine mammal stranding networks. Total body lengths were straight line measurements from the distal tip of the rostrum to the median notch on the trailing edge of the fluke (Geraci and Lounsbury, 1993).

Analyses

All analyses were performed using the statistical package R (R Core Team, 2015). Vaginal length, cumulative vaginal fold length, and total body length means were calculated for each species and base10 log-transformed to meet assumptions of a normal distribution and homogeneity of variance. Three regressions were conducted using phylogenetic generalized least squares (PGLS), implemented using the GLS procedure in the R package NLME (Pinheiro et al., 2015). Both mean vaginal lengths and mean cumulative vaginal fold lengths were regressed separately on mean total body lengths. The non-phylogenetically-controlled residual values of mean cumulative vaginal fold lengths were regressed on the non-phylogenetically-controlled residual values of vaginal lengths. The cetacean phylogenetic tree and branch lengths provided by McGowen et al. (2009) was used, and trimmed to remove

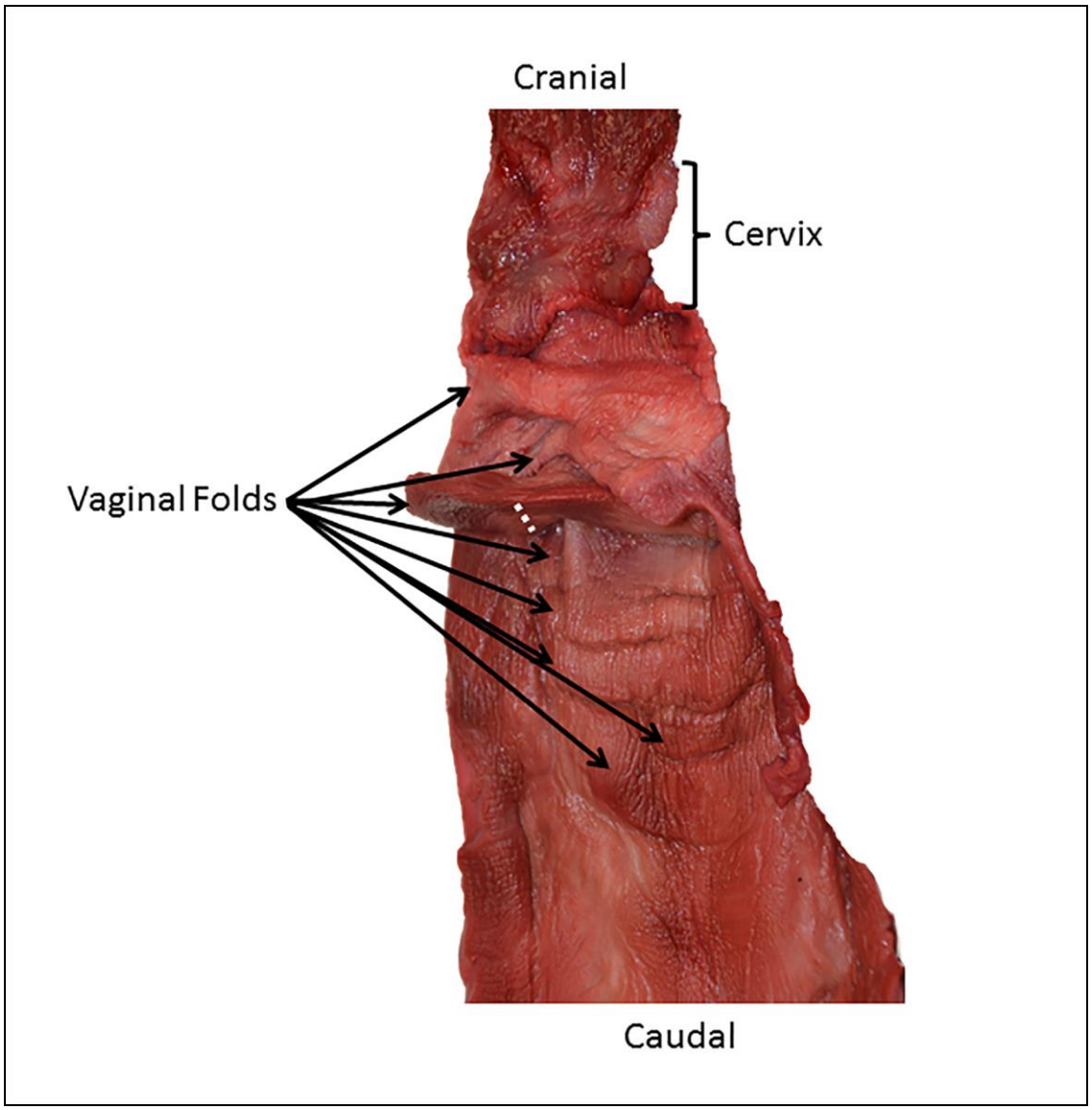

Figure 4-2. Vaginal fold length measurement of an adult female Pacific white-sided dolphin (*Lagenorhynchus obliquidens*). The black arrows point to eight vaginal folds in this specimen. Vaginal fold length was measured with a plastic scale positioned on the dorsal side of the fold. The cumulative measurements of vaginal fold length were straight lines from the base of the vaginal folds to their respective distal tips that projected into the lumen of the vagina. The white hashed line is positioned where one vaginal fold was measured.

species not in the database. A correlation structure (Pagel, 1999) was implemented using the CORPAGEL function in the R package APE (Paradis et al., 2004), as per Dines et al. (2015). The correlation structure estimates the extent to which trait variation is related to phylogeny using the parameter λ, which is robust to incomplete phylogenies (Freckleton et al., 2002). A λ value of 0 indicates phylogenetic independence, while a λ value of 1 indicates complete phylogenetic correlation (Pagel, 1999; Freckleton et al., 2002).

Results

A broad diversity in morphological measurements was found across the 58 reproductive tracts included in the analysis (19 species and 5 families; Table 4-1). Specimens varied in the number of vaginal folds (Table 4-1; Appendix A), which ranged from 1 (common bottlenose dolphins, *Tursiops truncatus*; long-beaked common dolphins, *Delphinus capensis*; short-beaked common dolphins, *Delphinus delphis*; Fig. 4-1) to 13 (harbor porpoises, *Phocoena phocoena*) (N = 58, mean ± SD = 4 ± 2.8). The mean vaginal length of specimens was 20.8 cm (N = 58, SD = 15.3). The mean cumulative vaginal fold length was 51.7 mm (N = 58, SD = 47.9). All vaginal folds greater than 1 mm projected caudally towards the external vaginal opening. Directionality could not be determined in vaginal folds less than 1 mm in length. In almost all specimens, vaginal folds were only present in the cranial half of the vagina. With the exception of some specimens in the genus *Phocoena*, *Kogia*, *Orcinus*, and *Lagenorhynchus*, vaginal folds generally decreased in length from the cranial to caudal direction.

A statistically significant and positive relationship was found when vaginal length was regressed on body length ($F = 11.287$, $R^2 = 0.257$, $df = 17$, $p = 0.004$, $\lambda = 0.82$; Fig. 4-3) and when cumulative vaginal fold length was regressed on body length ($F = 6.654$, $R^2 = 0.667$, $df = 17$, $p = 0.020$, $\lambda = 0.69$; Fig. 4-4). Larger species had longer vaginas and longer cumulative vaginal fold lengths compared to smaller species. Pygmy sperm whales (*K. breviceps*) had a particularly profound influence on the regression lines, as demonstrated by removing them from the dataset and rerunning the analyses. The cumulative vaginal fold length by vaginal length regression was not statistically significant ($F = 2.81$, $R^2 = 0.126$, $df = 17$, $p = 0.112$, $\lambda = 0.24$; Fig. 4-5). Species with the highest mean number of vaginal folds (Table 4-1) often had the longer relative vaginal fold lengths compared to species with the lowest mean number of vaginal folds (Figs. 4-4, 4-5).

Discussion

Vaginal length and cumulative vaginal fold length had a significant and positive relationship with body length and were correlated with phylogeny. However, vaginal length and cumulative vaginal fold length varied independently of each other. Selective forces appear to act differently on these two anatomical traits in cetaceans. Two non-mutually exclusive functional hypotheses are discussed- the prevention of the incursion of seawater into the reproductive tract (Slijper, 1962; Green, 1972, 1977; Chen et al.,

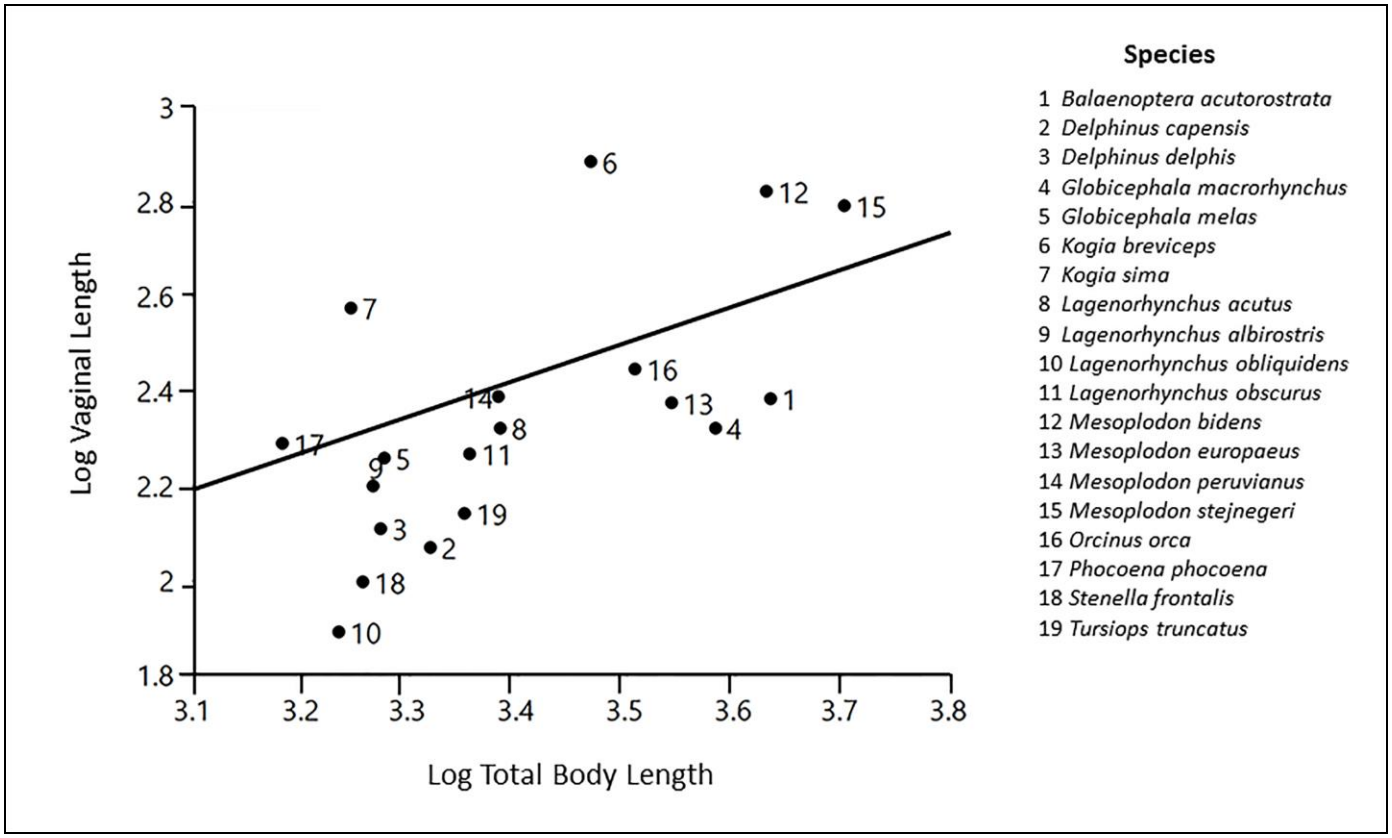

Figure 4-3. Regression of vaginal length on total body length. Vaginal lengths were measured from the base of the ectocervix to the cranial limit of the vulva. Total body lengths were provided by the marine mammal stranding networks that collected the reproductive tracts. Means were calculated for each species and data were base10 log-transformed. The solid line indicates the best fit line from a phylogenetic generalized least squares model. N = 19 species.

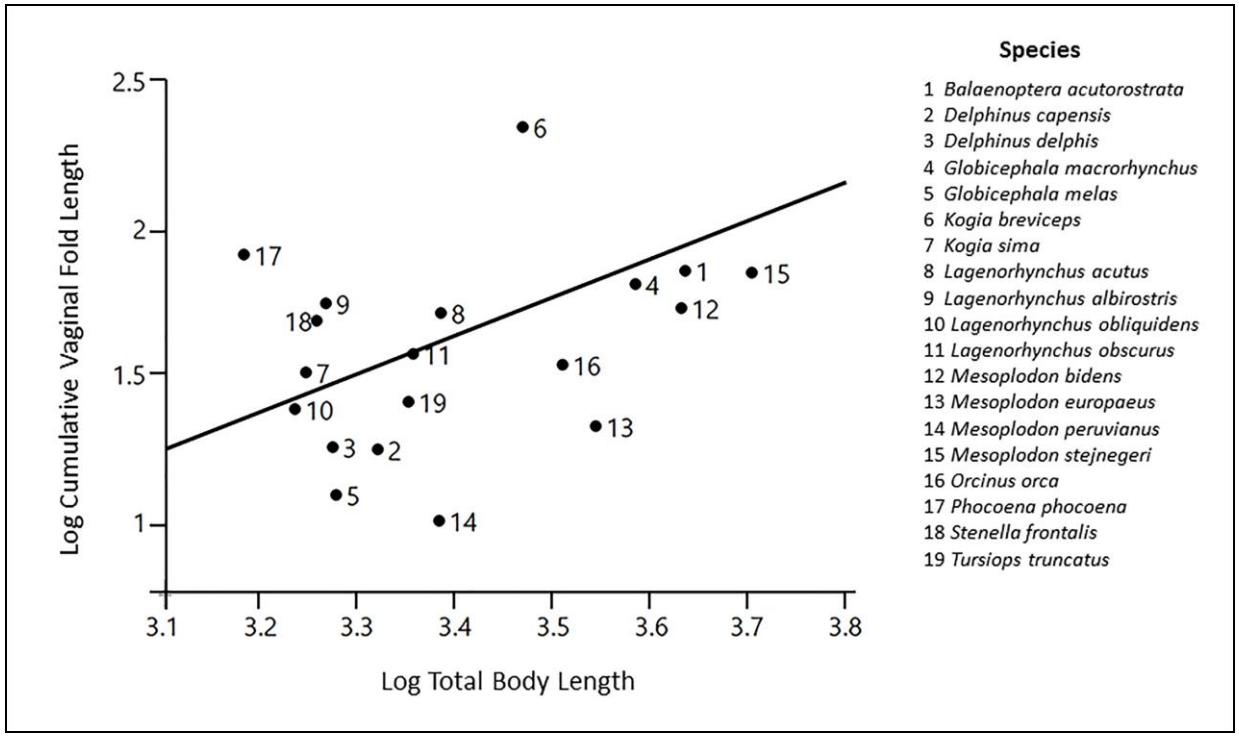

Figure 4-4. Regression of cumulative vaginal fold length on total body length. Vaginal fold lengths were measured from the base of each vaginal fold to its distal tip. Any fold length shorter than 0.5 mm was rounded to 1.0 mm. The sum of all vaginal fold lengths was tallied for each specimen to derive a cumulative value. Total body lengths were provided by the marine mammal stranding networks that collected the reproductive tracts. Means were calculated for each species and data were base10 log-transformed. The solid line indicates the best fit line from a phylogenetic generalized least squares model. N = 19 species.

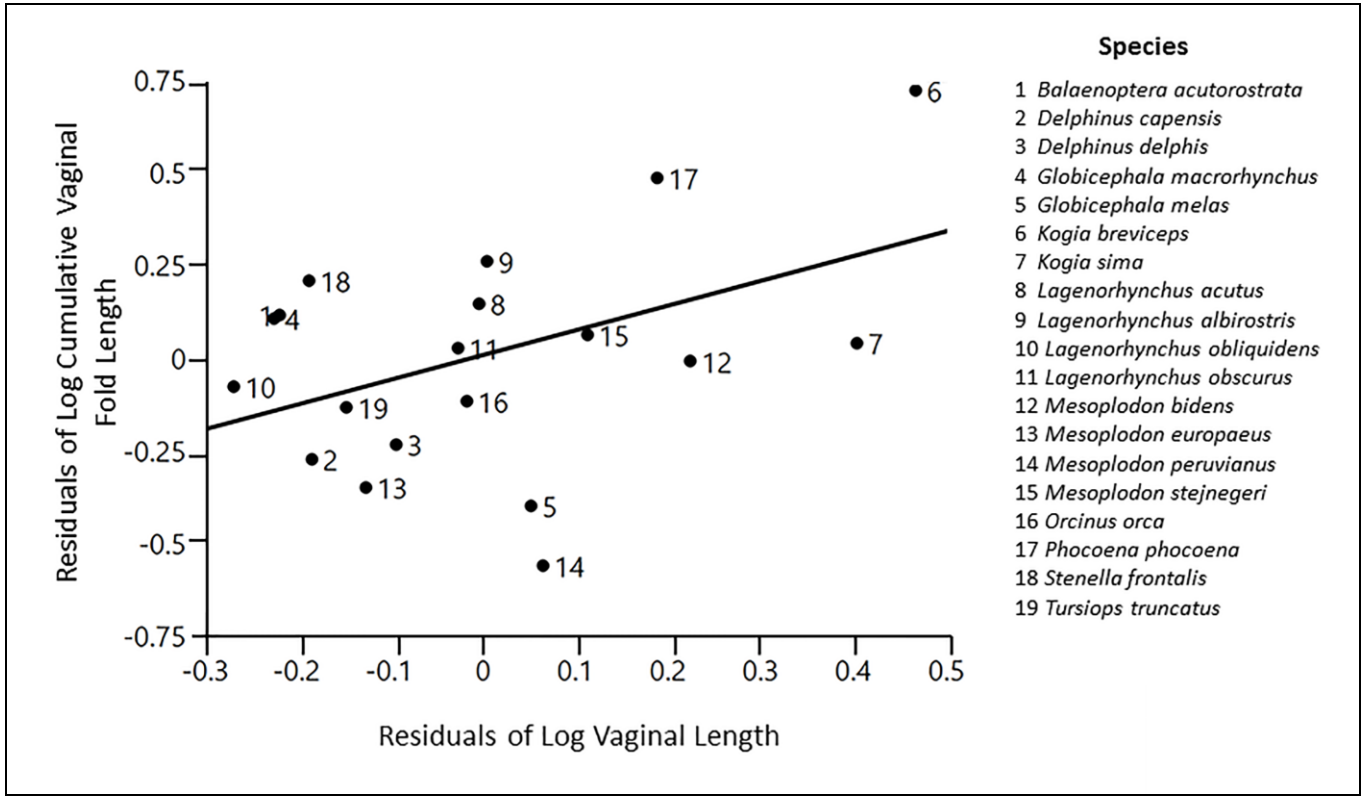

Figure 4-5. Regression of cumulative vaginal fold length on vaginal length. The non-phylogenetically controlled residuals of vaginal length on total body length and the non-phylogenetically controlled residuals of cumulative vaginal fold length on total body length were used. All raw vaginal length, vaginal fold length, and total body length data were base10 log-transformed. The solid line indicates the best fit line from a phylogenetic generalized least squares model. N = 19 species.

1984; Schroeder, 1990; Robeck et al., 1994) and post-copulatory sexual selection (Clarke et al., 1994; Orbach et al., 2016/chapter III).

Reproductive tract scaling (how genitals change in size relative to changes in body size) has been studied for males both within and among several taxonomic groups (reviewed in Kinahan et al., 2007). Male mammals tend to show significant positive allometric relationships (e.g., bats, Lüpold et al., 2004; harp seal, *Pagophilus groenlandicus*; Miller and Burton, 2001). However, few studies have investigated reproductive tract scaling in female mammals, and resulting patterns have been variable. Significant positive allometry was reported in Cape dune mole-rats (*Bathyergus suillus*; Kinahan et al., 2007), isometry was found in Hottentot golden moles (*Amblysomus hottentotus*; Retief et al., 2013), and no significant scaling patterns were found in Cape ground squirrels (*Xerus inauris*; Manjerovic et al., 2008) or chimpanzees (*Pan troglodytes*; Dixson and Mundy, 1994). However, these studies assessed patterns within rather than across species. Accordingly, there was no *a priori* assumption that cetacean vaginal morphology attributes would scale with body length. As vaginal length and cumulative vaginal fold length scaled significantly with body length, it was surprising that the association between these two variables (scaled by body length) was not significant. Since only 26% and 67% of the variance in vaginal length and cumulative vaginal fold length, respectively, were explained by body length, there must be additional explanatory factors.

The evolution of vaginal folds may reflect adaptive mechanisms developed to overcome challenges associated with living and mating in the marine environment.

Vaginal lengths and cumulative vaginal fold lengths could reflect different mechanisms for the same function– to prevent the flow of seawater by physical distances or physical barriers. Such "many-to-one mapping" of morphology to function is typical among a wide range of vertebrates (e.g., Wainwright et al., 2005). Since seawater is lethal to cetacean sperm (Schroeder and Keller, 1989), long vaginas may hinder the flow of water through the vaginal cavity. Long vaginas may thereby hamper the incursion of water into the cranial part of the vagina (where semen is deposited) and the upper reproductive tract (where fertilization occurs and the fetus develops in the uterine horn). Vaginal folds can form deep crypts (Table 4-1). They may function as physical barriers to seawater flow and might be particularly important in species with short vaginas. However, there are several lines of evidence that do not support this hypothesis. Species with short vaginas had short vaginal fold lengths (Fig. 4-5), although an inverse relationship between the two variables would be expected if vaginal folds form physical barriers. Similarly, species with few vaginal folds generally had shorter rather than longer relative vaginal fold lengths. Vaginal folds were usually concentrated at the cranial end of the vagina rather than distributed evenly through its length, although seawater could also be present in the caudal vagina. As it is commonly thought that all water (including freshwater) is lethal to mammalian sperm (e.g., Holt, 2000; Santos et al., 2011), the presence of vaginal folds in a river dolphin that inhabited only freshwater environments does not preclude the barrier to water functional hypothesis (baiji, *Lipotes vexillifer*; Chen et al., 1984). However, if vaginal folds serve as physical barriers to water flow, it is unclear why other non-cetacean marine mammals that mate only in the water lack vaginal folds (phocids;

Harrison et al., 1952; Amazonian manatees; Rodrigues et al., 2008; sea otters; Sinha et al., 1966). Collectively, these observations suggest that other factors are needed to explain the variability in the presence and development of vaginal folds in cetaceans.

Cetaceans may have other barriers that occlude seawater from entering their reproductive tracts and affecting osmoregulation. The lack of vaginal fold-like structures in non-cetacean marine mammals may indicate that the tight vaginal seal of the *labia minora* may be sufficient to prevent seawater and marine debris from entering the reproductive tract, including during non-mating contexts. Any seawater that overcomes the physical barrier of the *labia minora* may encounter additional challenges that prevent entry into the cranial vagina or upper reproductive tract. For example, the hymeneal folds of seals are located more caudally in the vagina compared to cetacean folds and could potentially form a physical barrier (Atkinson, 1997). The cervix functions as a critical barrier to the upper reproductive tract. The anti-microbial defenses and immune responses of the vagina and cervix, in addition to cervical mucus, can prevent movement of foreign bodies into the upper reproductive tract (reviewed in Suarez and Pacey, 2006). The thick cervical mucus and narrow aperture and passageway of the endocervix observed in cetaceans appear to largely occlude the opening of the uterus (Slijper, 1962; Orbach et al., 2016/chapter III). Future studies that investigate the relationship between vaginal length, fold complexity, and cervical conformation are warranted.

Vaginal modifications could also be shaped by post-copulatory sexual selection and the coevolution of male and female reproductive anatomy (Eberhard, 1996; Hosken and Stockley, 2004; Brennan et al., 2007). Vaginal folds might be concentrated in the

cranial half of the vagina because ejaculation occurs proximate to the cervix and distal to the vaginal orifice (T. Robeck, pers. comm.). Since the distal tips of vaginal folds projected caudally within the lumen of a vagina and generally decreased in size from cranial to caudal direction, like a funnel, they may restrict sperm from entering the cranial vagina or upper reproductive tract rather than retention. Future studies that use *in vivo* vaginal endoscopy of recently mated cetaceans or physical models might be able to illuminate whether vaginal folds form physical barriers to seawater and sperm movement; this can be accomplished by examining and distinguishing where seawater and sperm pool within the vagina or vaginal model.

Assessment of the relationship between vaginal complexity and testes size or penile morphology (these two male traits are correlated in baleen whales; Dines et al., 2014) could provide evidence of a role of vaginal folds in sexual selection. For example, oviduct length was positively correlated with testes weight and with sperm characteristics across 33 genera of mammals (Anderson et al., 2006). Only three species- pygmy sperm whales (*Kogia breviceps*), dwarf sperm whales (*K. sima*), and harbor porpoises (*Phocoena phocoena*)- were consistently located above the body size regression line for both vaginal length (Fig. 4-3) and cumulative vaginal fold length (Fig. 4-4). These three species all engage in sperm competition, as supported by their large relative testes sizes (Dines et al., 2015). Studies of how penile morphology relates to vaginal fold shape, length, or positioning, and how deep the penis penetrates the vagina and possibly the cervix during copulation are needed to further explore the sexual selection functional hypothesis of vaginal folds.

The data demonstrate that vaginal length was not a significant predictor of cumulative vaginal fold length. This may be because the two characters function differently to restrict seawater and/or semen to the caudal vaginal region (i.e., physical distance and physical barrier). Cumulative vaginal fold length was selected as a variable because it was a straightforward and quantitative measure of the extent of obstruction of the vaginal lumen. However, other features of vaginal folds, such as thickness, shape, or number– as well as other features of the entire reproductive tract, such as the lengths of various chambers- could be informative in determining function. Although data were not obtained for all 90 extant cetacean species, the data are representative of 10 genera across the cetacean phylogeny. The data provide a robust indication of taxon-wide patterns that can be further expanded to advance our understanding of the evolution of genital morphology.

The data present the most comprehensive compilation of vaginal fold size diversity across cetaceans. Although vaginal folds have been reported in several cetacean species (e.g., Schroeder, 1990), quantitative measurements of vaginal morphology were not collected for most species, nor measured using consistent landmarks. This lack of information has hindered the ability to systematically assess the function(s) of vaginal folds in cetaceans. Although both vaginal length and cumulative vaginal fold length scaled significantly with body length, no relationship was found between them. Since not all variance in the regressions was explained by body size and the factors were not predictors of each other, vaginal folds may have function(s) that are maintained by natural and/or sexual selection pressures. This study lays the foundation

for tests of functionality that will determine if vaginal folds are an example of specialized adaptations to aquatic living and/or relate to sexual selection.

CHAPTER V

REPRODUCTIVE MORPHOLOGY AND FEMALE MATING BEHAVIORS IN DOLPHINS AND PORPOISES*

Summary

Sexual selection can strongly influence genital diversity and mating behaviors, yet the integrated coevolution of reproductive anatomy and mating behavior between the sexes has received little attention. Studies of trade-offs between investments in pre- and post-copulatory traits can enhance the understanding of sexual selection mechanisms. Using a comparative approach, variation in female/male post-copulatory traits (vaginal complexity/relative testes size) was used to predict female pre-copulatory traits (behavior repertoire size and intensity) within and between three species of toothed whales. Female pre-copulatory effort was predicted to be highest in Indo-Pacific bottlenose dolphins (*Tursiops aduncus*), intermediate in dusky dolphins (*Lagenorhynchus obscurus*), and lowest in harbor porpoises (*Phocoena phocoena*). Video recordings of mating events in free-ranging populations were analyzed. The size of female behavioral repertoires did not vary across the three species, and female Indo-Pacific bottlenose dolphins exhibited more low intensity behaviors while dusky dolphins exhibited more high intensity behaviors than expected by chance. As female behavioral

*This chapter is intended for publication in collaboration with Christopher Marshall, Sarah Mesnick, and Bernd Würsig.

patterns were inconsistent with predictions based on reproductive anatomy, it is possible that vaginal complexity is not costly to maintain, alternative measures of female control over paternity are warranted, or socio-sexual and physical environmental factors have a large influence on behavior. Application of the comparative approach highlights the behavioral diversity of dolphins and porpoises and that females may use a variety of pre- and post-copulatory mechanisms to control paternity.

Introduction

The conceptual framework of the evolution of mating strategies should be broadened by shifting the focus on male competitive strategies to encompass the coevolution of both sexes (Bro-Jørgensen, 2011). The wide diversity in male genital morphology is commonly attributed to sexual selection (Eberhard, 1985; Arnqvist, 1998; Hosken and Stockley, 2004), and has often been linked to the evolution of male mating behavior. For example, the relationship between relative testes size (commonly used to gauge sperm production) and mating system has been explored in many taxa (Smith, 2012), including fish (Neff et al., 2003), reptiles (Aldridge, 1993), birds (Møller and Briskie, 1995), and mammals (Harcourt et al., 1981; Heske and Ostfeld, 1990; Dines et al., 2015). The correlated evolution of genitalia between the sexes has been demonstrated (Arnqvist and Rowe, 2002a; Hosken and Stockley, 2004; Brennan et al., 2007; Rönn et al., 2007; Kuntner et al., 2009; Tatarnic and Cassis, 2010; Simmons and Garcia-Gonzalez, 2011; Tanabe and Sota, 2014), including in mammals (Anderson et al., 2006). Yet the dynamic coevolution of pre-copulatory traits (e.g., mating behavior) and post-copulatory traits

(e.g., reproductive morphology) between the sexes has received considerably little attention, largely because female genital form and importance in sexual selection have been overlooked (Ah-King et al., 2014).

However, the coevolution of male and female pre- and post-copulatory traits has been documented within only a few taxa. For example, in water strider (*Gerris* sp.) species with male armaments that enhanced clasping abilities of females during copulation, females had anatomical counter-adaptations that reduced male grip (Arnqvist and Rowe, 2002a). Mating rate frequency and duration reflected which sex had more exaggerated armaments (Arnqvist and Rowe, 2002b). Similarly, male waterfowl have an anti-clockwise shaped phallus, the length of which was positively correlated with the number of clockwise vaginal spirals (Brennan et al., 2007). These vaginal spirals appear to prevent males from everting their phallus deep into the vagina during forced-extra pair copulations, when females struggle and do not relax vaginal muscles (Brennan et al., 2010). The correlated evolution of pre- and post-copulatory traits between the sexes has not been explored in whales, dolphins, and porpoises.

Cetaceans are a good model system in which to explore the relationship between the sexes in pre- and post-copulatory traits. There is broad variation in vaginal complexity and testes size across the taxa that is not adequately explained by body size or phylogenetic relatedness, but may be accounted for by post-copulatory sexual selection (chapter IV; appendix A; Dines et al., 2015). Males exhibit trade-offs between investments in pre- and post-copulatory traits (Connor et al., 2000a; Dines et al., 2015) that may also occur in females (Wong and Candolin, 2005). Additionally, both sexes

display diverse mating tactics/behaviors (chapter I) that can elicit different behavioral responses from the other sex (Orbach et al., 2015a/chapter II). Furthermore, mating behavioral events are overt, varied, and can be separated into discrete quantifiable units (Orbach et al., 2015a/chapter II).

The goal of this chapter is to explore the variation in female and male post-copulatory traits, and female pre-copulatory traits, within and between three species of toothed whales. Specifically, this chapter: 1) examines species-specific differences in female and male post-copulatory traits (vaginal complexity/testes size), 2) predicts variation in female pre-copulatory traits (behavioral effort to evade males) based on female and male post-copulatory traits, and 3) explores species-specific disparities in female pre-copulatory traits using video recordings of mating events. Females are predicted to have simple vaginal morphologies in species with small relative testes sizes, and conversely, complex vaginal morphologies in species with large relative testes sizes. If so, then an inverse relationship between investments in post-copulatory traits and female pre-copulatory traits is predicted.

Materials and Methods

Post-copulatory Traits

Reproductive anatomical measurements were collected from fresh or moderately decomposed (less than 24 hour post-mortem) deceased dolphins and porpoises. Excised female reproductive tracts were frozen and transferred to necropsy facilities located at Texas A&M University at Galveston or the National Oceanic and Atmospheric

Administration's (NOAA) Southwest Fisheries Science Center for dissections. The reproductive tracts were collected under a National Marine Fisheries Service (NMFS) salvage permit letters and an institutional Convention on International Trade in Endangered Species of Wild Fauna and Flora permit (CITES Mammal Import Permit: 15US774223/9; MMPA/ESA research permit: 14097). Male measurements were collected directly by the responding marine mammal stranding networks.

For each species, data from both sexes were collected from the same or nearby populations to control for potential variation in reproductive morphology across populations (e.g., Kelley et al., 2015; Orbach et al., 2016/chapter III). Common bottlenose dolphin (*Tursiops truncatus*) specimens and data were obtained from the Texas Marine Mammal Stranding Network (Galveston, Texas; $N_{Females} = 5$, $N_{Males} = 5$). Dusky dolphin (*Lagenorhynchus obscurus*) specimens and data were obtained from the New Zealand Common Dolphin Project at Massey University (Auckland, New Zealand; $N_{Females} = 3$, $N_{Males} = 3$). Fresh female harbor porpoise (*Phocoena phocoena*) specimens were obtained from The Marine Mammal Center (San Francisco, California; $N_{Females} = 8$), while data from males were obtained from Toperoff (2002; Monterey Bay, California; $N_{Males} = 3$). The marine mammal stranding networks that collected the reproductive tracts provided data on the date and location of each stranding, sexual maturity state, total body length, and gonad weight (for males). Only sexually mature specimens were included in the analysis. Sexual maturity was defined by the presence of *corpora lutea* and/or *corpora albicantia* on either ovary (Ivashin, 1984), or by asymptotic body length (e.g., Mallette et al., 2016). Additionally, only males that

stranded during the peak breeding season for their respective population were included in the analysis, due to potential seasonal swelling and recession of the testes (Cipriano, 1992; Neimanis et al., 2000; Murphy et al., 2005; Kelley et al., 2015).

Morphological complexity can be difficult to define and measure (McShea, 2000). Two measurements were used as proxies of vaginal complexity per species- the mean number of vaginal folds and the mean cumulative vaginal fold length scaled by body size. Both measurements were calculated for each female specimen following the protocol in Orbach et al. (2016/chapter III; chapter IV). The number of vaginal folds was a standardized count of fold-like structures (Fig. 5-1). Only vaginal folds exceeding 5 mm in length were included, as smaller folds could not consistently be distinguished from smooth vaginal wall tissue (Orbach et al., 2016/chapter III). The cumulative vaginal fold length was the sum of measurements from the base of each vaginal fold to its distal tip that projected into the lumen of the vagina. Total body length, from the distal tip of the rostrum to the median notch on the trailing edge of the fluke (Geraci and Lounsbury, 1993), was provided by the marine mammal stranding networks. The mean number of vaginal folds, mean cumulative vaginal fold length, and mean total body length were calculated for each species. Mean cumulative vaginal fold length was regressed on mean total body length; the larger database and phylogenetic generalized least squares statistical approach from chapter IV were used to derive a single residual value for each species. All data were base10 log-transformed prior to substitution into the larger database as per MacLeod (2010).

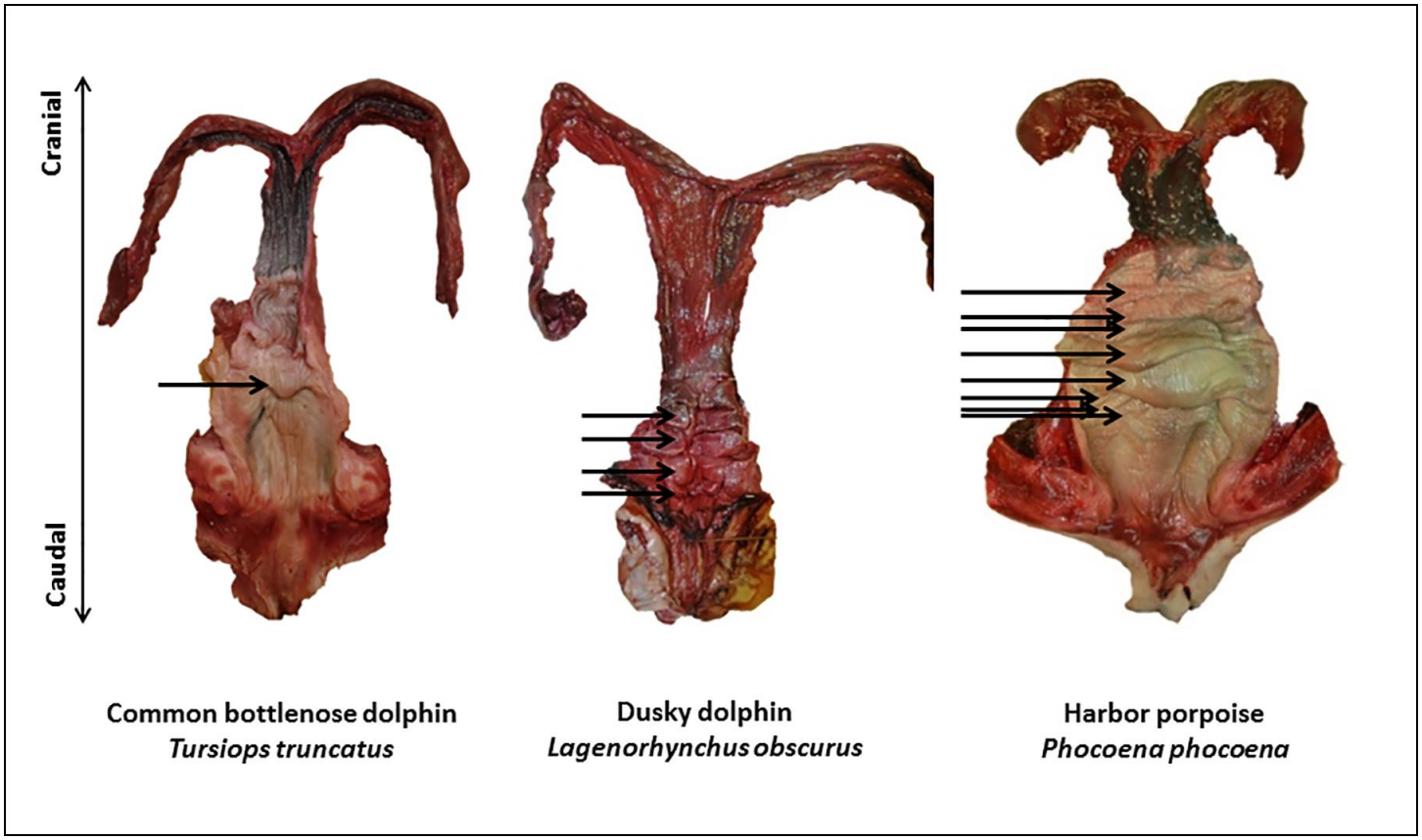

Figure 5-1. Number of vaginal folds in the dissected reproductive tracts of a common bottlenose dolphin, dusky dolphin, and harbor porpoise. The specimens are oriented in dorsal recumbency. The black arrows denote vaginal folds above 5 mm in length from their base to distal tip.

One measurement, mean testes mass scaled by body size, was used as a proxy for male post-copulatory investment in sexual selection. The left and right combined testes mass with the epididymis attached and the total body length data were provided by the marine mammal stranding networks. The mean testes mass and mean total body length were calculated for each species. Mean testes mass was regressed on mean total body length; the larger database and phylogenetic generalized least squares statistical approach from Dines et al. (2015) were used to derive a single residual value for each species. All data were base10 log-transformed prior to substitution into the larger database. The mean testes mass was used instead of the maximum testes mass as anomalous outliers could misrepresent the species. The residual values calculated using mean and maximum testes masses yielded the same pattern for the three focal species, indicating that the mean testes mass data were appropriate representations of the species.

Female and male post-copulatory traits were plotted using JMP Pro 12.0.1 (SAS Institute Inc., Cary, NC). The two measures of vaginal complexity- mean number of vaginal folds and residuals of cumulative vaginal fold length (scaled by body length)- were plotted separately against the residuals of testes mass (scaled by body length). Only data from common bottlenose dolphins, dusky dolphins, and harbor porpoises were included.

Study Populations and Recording Techniques

Indo-Pacific Bottlenose Dolphins (T. aduncus)

The mating behaviors of Indo-Pacific bottlenose dolphins, a congener of common bottlenose dolphins (*T. truncatus*), were analyzed as a proxy due to extensive logistical

difficulties locating a population of cetaceans with conditions favorable for frequent and systematic observations of mating events (reviewed in Lanyon and Burgess, 2014). A population of >200 Indo-Pacific bottlenose dolphins is found year-round in the Red Sea, along the coastline of Hurghada, Egypt (27°1” N, 33°55” E). Males are slightly larger than females (Wilson and Mittermeier, 2014). Mating behaviors peak during spring and summer, although the dolphins can be observed mating throughout the year. Small and temporarily stable mating groups form in the larger aggregation of dolphins. Males have more stable association patterns than females and form pairs or quads (two pairs together) that can last for seasons and years, potentially indicating coalition or alliance formation (Dolphin Watch Alliance, unpublished data). Extended consortships with females and coercive mating might also be prevalent male mating tactics, as males have been observed following sleeping females and chasing after resistant females (Angela Ziltener, pers. comm.). Alliance formations, extended consortships, and coercive matings have been reported in some other populations of Indo-Pacific bottlenose dolphins (e.g., Shark Bay, Western Australia; Connor et al., 1992a, 2000b; Connor and Smolker, 1996).

The underwater mating interactions of Indo-Pacific bottlenose dolphins were filmed opportunistically between 2012 and 2015 in the months of December through June and August. Videos were recorded by Angela Ziltener and collaborators of Dolphin Watch Alliance, who have been studying the population since 2009 (Kleinertz et al., 2014). Typical water visibility was 30 m. Dolphins were surveyed from a research vessel for a minimum of 5 min, during which the group size, group behavior, and group

composition were recorded. Mating groups were defined as those in which the male extruded his penis, side-mounting another dolphin (slid his ventrum along the side of the other dolphin or over the dorsal region of another dolphin, forming a 't'-shaped formation; Connor et al., 2000b), and hooked his penis under the ventrum of the other dolphin. A minimum of two scuba divers entered the water and video recorded the behaviors of the mating groups during *ad libitum* focal follows. Continuous videos were made with a Panasonic Lumix DMC-TZ7, Garmin VIRB, or Sony Cyber-shot DSC-RX100 recorder. The sex and age classes of the dolphins were determined underwater by observations of the uro-genital slit and/or penis, body size, and amount of speckling on the dolphin's ventrum (Krzyszczyk, 2013). Any mating groups without a confirmed female were excluded from the analysis. Mating group follows were terminated when sexual behaviors stopped, typically when the animals surfaced to breathe. No research permit was required, although authority was provided by Red Sea Governor of Egypt and *Technische Universität Berlin* Campus El Gouna.

Dusky Dolphins (L. obscurus)

The population of dusky dolphins in the waters off Kaikoura, New Zealand (42°25"S 173°41"E) consists of >13,000 individuals that form large aggregations with highly fission-fusion social structures (Markowitz, 2004; Würsig et al., 2007). Approximately 2,000 dusky dolphins are present off Kaikoura at any given time (Markowitz, 2004). Females and males are sexually monomorphic in body size (Cipriano, 1992). Foraging

off Kaikoura occurs offshore during the night on the deep scattering layer of mesopelagic myctophids and squids (Benoit-Bird et al., 2004, 2009) and is rarely observed during the day (Markowitz, 2004, 2012). Predominant daytime behaviors consist of resting and socializing (Markowitz, 2004). Mating behaviors peak during austral summer, when there is also a seasonal increase in the testes size of adult males (Cipriano, 1992; Würsig et al., 1997; Markowitz, 2004). The predominant mating tactics of males are sperm competition (Cipriano, 1992) and exploitative scramble competition (Orbach et al., 2014, 2015b). Labile near-shore mating groups with a mode group size of four adult males and one adult female form near larger groups (Orbach et al., 2014).

The surface behaviors of mating groups were recorded during daylight hours between October 2013 and January 2014. The water visibility was typically 5-8 m (range 2-15 m). Mating groups were identified by observations of re-orientation leaps, copulation attempts, and the presence of males swimming inverted with their penises extruded (Markowitz, 2004; Markowitz et al., 2010; Orbach et al., 2014). Mating groups were followed from a 6-m rigid-hull inflatable vessel with an 80-hp 4-stroke outboard engine. Groups maintained constant membership and individuals were within 10 m of each other for the duration of the focal follow (Smolker et al., 1992). Group size and composition were recorded for each focal group follow. Continuous videos were recorded using a Sony Handycam HDR-XR550V stabilized on a chest-pod while travelling parallel and within 20 m (mean ~ 5 m) distance of the groups. The female in the group was continuously tracked. She was identified as the nuclear animal that repeatedly had inverted males with their penises extruded beneath her. As the female

was characteristically ventrum-down at the surface, she could be identified and tracked after resurfacing from a dive by her individually distinctive dorsal fin marking(s). Each mating group follow ended when mating behaviors ceased, group size changed, or boating conditions became unsafe. No research permit was required, although authority was provided by the New Zealand Department of Conservation.

Harbor Porpoises (Phocoena phocoena)

Harbor porpoises re-inhabited San Francisco Bay (37°71”N, 122°28”W) in 2007, following a 65 year absence (Keener et al., 2011a). Over 700 harbor porpoises use the San Francisco Bay (Golden Gate Cetacean Research, unpublished data) of the larger San Francisco-Russian River stock (9,886 individuals; Forney et al., 2014). Harbor porpoises are typically solitary animals or form labile groups of two individuals (e.g., Forney et al., 2014). Slight reverse sexual size dimorphism is common in the species (Read and Tolley, 1997). Harbor porpoises move in and out of San Francisco Bay following tidal flows, with the highest number of animals accumulating near the Golden Gate Bridge within three hours of maximum inflow current velocity (Duffy, 2015). Mating is observed year-round beneath the Golden Gate Bridge (Keener at al., 2011a), although data from other populations indicate that conception is seasonal and peaks in spring and early summer (Read and Hohn, 1995; Fontaine and Barrette, 1997; Neimanis et al., 2000). Based on extensive seasonal enlargement of the testes, the primary male mating tactic of harbor porpoises is presumed to be sperm competition (Fontaine and Barrette, 1997; Neimanis et al., 2000). However, observations of harbor porpoise mating events in

San Francisco Bay suggest males may also use an alternative reproductive tactic (ART); males aggregate near the Golden Gate Bridge around ebb tide and intercept females following the tide cycle out of San Francisco Bay (pers. obs.). Although males do not appear to be parasitizing on each other's efforts to attract females, this pattern of intercepting females is similar to the satellite/sneaker tactics found in some other species (e.g., fish, Taborsky, 1994; natterjack toads, *Bufo calamita*: Arak, 1998).

The surface mating behaviors of harbor porpoises were filmed from the Golden Gate Bridge in collaboration with William Keener of Golden Gate Cetacean Research. The Golden Gate Bridge is 1,600 m long, 69 m above sea level at center span (U.S. Coast Pilot, 2016), and serves as a stationary aerial platform where porpoises can be non-invasively observed from the public walkway (Keener et al., 2011b). Videos were filmed opportunistically during December 2012, April 2014, April 2015, November 2015, and December 2015, with a Sony Handycam HDR-XR550V video camera fitted with a 1.7x teleconversion lens (Sony VCL HG1737C) or with a Canon 7D dSLR camera fitted with a 300 mm fixed lens. The group size and composition were recorded for mating groups. Mating groups were identified by the presence of a male that swam rapidly by a female or leaped aerially (umbilicus out of the water) with his body adjacent or in physical contact with a female. The penis was usually extruded during these events, which confirmed the animal's sex. However, it was not always possible to confirm if the penis was extruded based on the angle of orientation to the harbor porpoises. Females were identified by their consistent ventrum-down body positioning at the commencement of a copulation attempt or by their physical proximity to a calf. Mating

group follows terminated when the male and female separated. No research permit was required for this observational study.

Video Analysis

Two female pre-copulatory traits- behavioral repertoire size and behavioral intensity- were used as measures of female behavioral effort during mating interactions and contrasted across species. Detailed male behaviors were not assessed in this study because of difficulties continuously tracking multiple males simultaneously and recording all male behavioral events. Additionally, male dusky dolphins were inverted beneath females and male harbor porpoises were sub-surface and out of view prior to copulation attempts, which restricted abilities to observe and record male behaviors. Video recordings of mating events were analyzed using the software Transana (Woods and Fassnacht, 2015), where the frame rate could be slowed down by 10x. Each video corresponded to a single focal follow of a mating group, and could contain multiple copulation attempts. The videos were played back frame-by-frame and all occurrences of male and female behaviors were transcribed and time stamped. Within each species, the number of occurrences of each female behavior type was tallied and pooled across videos to facilitate interspecific comparisons (Table 5-1). The list of female behavior types (Table 5-1) was developed after reviewing several videos of mating groups for each species, and incorporated previously defined behaviors (e.g., Östman, 1985; Mann and Smuts, 1999; Markowitz et al., 2010). Only behavior types that were recorded on at least three occasions were

Table 5-1. Repertoire of female behaviors observed in mating groups for three species. The behaviors are categorized by their type and further divided by species (*Ta = Tursiops aduncus*; *Lo = Lagenorhynchus obscurus; Pp = Phocoena phocoena*). The occurrences of behavioral events, corresponding behavioral intensities, and definitions are included.

Behavior Type	Species	No. of Events	Behavioral Intensity	Definition
Tail slap	*Lo*	453	High	Female raises her tail out of the water and strikes it against the surface of the water with force, creating a noisy splash.
Direction change (veer)	*Lo*	179	High	Female quickly moves non-linearly through the water at the surface, abruptly switching direction one or more times.
Direction change (gradual)	*Ta* *Pp*	170 5	Low	Female slowly moves non-linearly through the water at or below the surface, progressively switching direction one or more times without losing momentum. The change in direction could be within 1 plane (i.e., horizontal or vertical) or across planes (i.e., horizontal and vertical)
Body roll	*Ta* *Lo* *Pp*	135 150 16	Low	Female rotates her body along her longitudinal axis (e.g., rolls onto her back).
Re-orientation leap	*Lo*	54	High	Female leaps vertically out of the water and re-enters head-first nearby. Her ventral surface is oriented down. Her entire body clears the surface of the water and no loud splash is generated.

Table 5-1, Continued.

Behavior Type	Species	No. of Events	Behavioral Intensity	Definition
Accelerated swim	*Ta* *Lo*	13 15	High	Female moves horizontally and at high speed with minimal changes in direction. The behavior is usually accomplished by exaggerated undulations of the fluke.
Spy hop	*Lo*	12	Low	Female stops swimming forward and changes body positioning from horizontal to vertical. She half-rises vertically out of the water, with at least her eyes and rostrum above the surface.
Passive	*Ta* *Pp*	38 5	Low	Female demonstrates no behavioral reaction and is listless (floating) or continues her normal slow swimming pattern.
Dive	*Pp*	16	High	Female that was at the surface re-positions her body rostrum-down and swims vertically away from the water's surface while moving her fluke rapidly to overcome inertia.
Open Mouth	*Ta*	9	Low	Female's mouth is ajar with a distance between the maxilla and mandible of at least the height of the maxilla
Peduncle Curl	*Ta* *Pp*	3 13	Low	Female contracts muscles to slowly lower her caudal peduncle (tail) so that her body forms a "J" shape. She has limited movement of her flukes and while her peduncle is curled down.
Fluke Lift	*Pp*	6	High	Female extends her fluke straight in the air with force, creating a splash. Her body is arched back.

included in the analyses. The duration of mating group interactions and the number of copulation attempts per minute (group behaviors) were calculated per species. Since it was not possible to confirm if successful intromission and ejaculation occurred, all mating events in which the male aligned his penis with the uro-genital opening of the female were termed 'copulation attempts'. It was assumed that different focal females were present in each video, all females were sexually mature, all females were in the same state of estrus, and each behavior exhibited by a female was independent of other behaviors.

Variation in female behavioral repertoires was assessed across and within species. Repertoire size was a quantitative measure of the diversity of female behaviors observed during pre-copulatory mating sequences. The observational unit was all occurrences of a female behavioral event. To compare behavioral repertoire sizes across species, the number of behavior types listed in Table 5-1 was counted separately for each species. To understand the variation in female behaviors within a given species, we tested if each behavior type had an equal probability of occurrence. The number of occurrences of each behavior type was converted to a frequency for each species. The probabilities of occurrence were then assessed separately for each species using chi-square goodness of fit tests, based on the equation in Lehner (1996). All tests were performed in Microsoft Excel (2010).

Variation in the relative intensity of female behavior types was compared across species. The behavior types in Table 5-1 were qualified as high or low intensity. High intensity behavior types were likely low in energy efficiency (e.g., leaps, Blake, 1983;

Yazdi et al., 1999), while low intensity behavior types were associated with comparatively less energy loss (e.g., open mouth). The subjective nature of qualifying the intensity of behaviors was mitigated using an inter-rater reliability approach. Three cetacean researchers, each with extensive experience observing at least one population in this study, independently qualified the behavior types as high or low intensity using only the definitions provided in Table 5-1. All behavior types were scored concordantly by all three researchers. It was then tested if female behavioral occurrences had an equal probability of being high or low intensity events, given that a behavioral event was observed in a given species. A log-likelihood ratio test (G^2; equation from Bishop et al., 1975) was implemented in Microsoft Excel (2010). The counts of high intensity behavioral occurrences were pooled across behavior types within each species, as were the counts of low intensity behavioral occurrences. For example, female dusky dolphins exhibited 150 body rolls and 12 spy hops, for a total of 162 low intensity behavioral events (Table 5-1). Binomial z-scores were calculated using a threshold score of 1.96 (Bakeman and Gottman, 1986) to determine which behavioral intensity occurred at a higher or lower frequency than expected by chance. It was assumed that behavioral occurrences were independent, behavior types were mutually exclusive, and the expected frequencies of each variable were above 5.

Results

Post-copulatory Traits

Whether the mean number of vaginal folds (Figs. 5-1, 5-2A) or the mean cumulative vaginal fold lengths (Fig. 5-2B) were used as a proxy of vaginal complexity, common bottlenose dolphins (*T. truncatus*) had a simple vaginal morphology and small testes size compared to harbor porpoises (*P. phocoena*) and dusky dolphins (*L. obscurus*). Harbor porpoises had the most complex vaginal morphology and intermediate testes size of the three species, while dusky dolphins had intermediate vaginal complexity and the largest testes size. Female Indo-Pacific bottlenose dolphins were predicted to have the largest behavioral repertoire size and display the highest behavioral effort in mating groups, based on the vaginal complexity and testes size of the congeneric species, and the expected trade-offs between pre- and post-copulatory investment in sexual selection. Female harbor porpoises were predicted to have the smallest behavioral repertoire size and display the lowest behavioral effort because they invested the most in post-copulatory sexual selection (based on vaginal complexity). Female dusky dolphins were predicted to have an intermediate behavioral repertoire size and level of behavioral effort because they had an intermediate degree of vaginal complexity.

Video Analysis

The durations of mating group interactions, frequencies of copulation attempts, and mating group sizes are presented in Table 5-2. Videos of 16 Indo-Pacific bottlenose dolphin groups corresponded to 1465 sec of mating interactions, with a mean duration

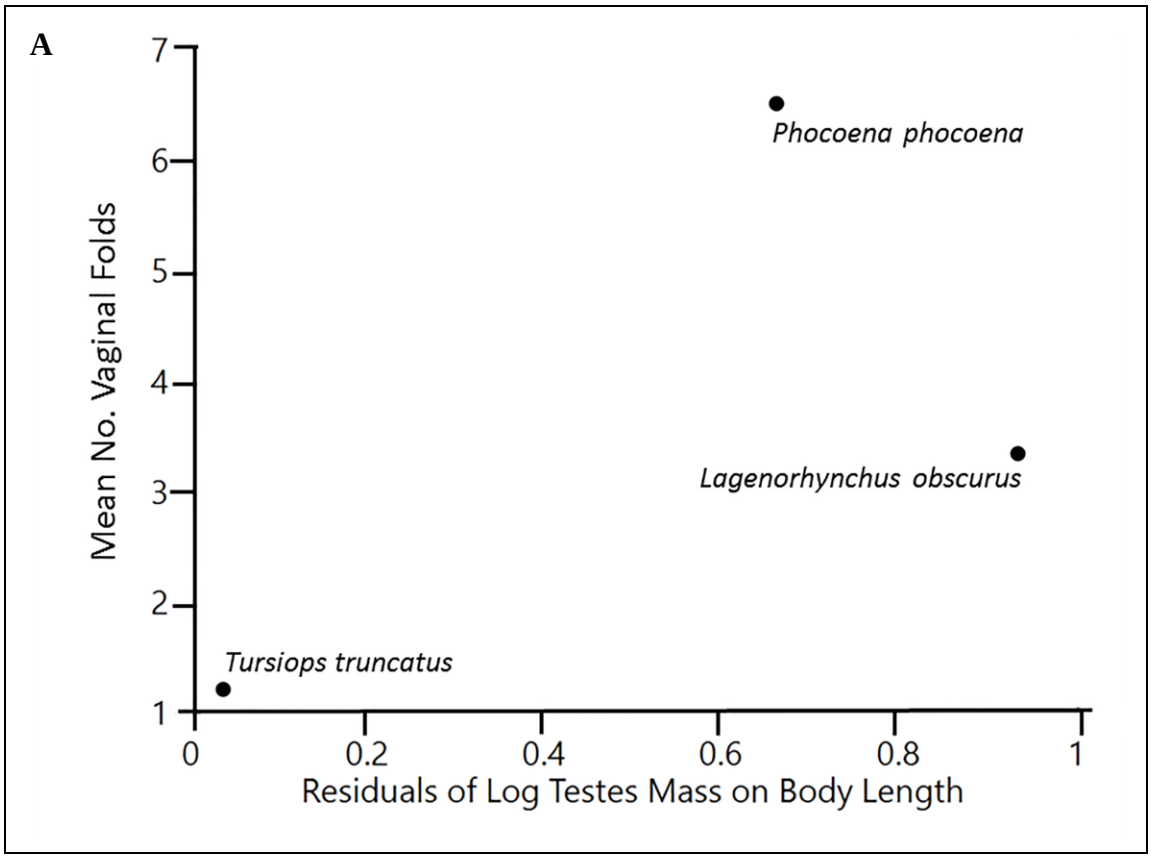

Figure 5-2. Post-copulatory traits (vaginal complexity and testes size) in common bottlenose dolphins, dusky dolphins, and harbor porpoises. Two measurements were used for vaginal complexity following the protocol in Orbach et al. (2016/chapter III, chapter IV): (A) number of vaginal folds, and (B) cumulative vaginal fold lengths. The residual values in B were calculated by substituting data into the larger database in chapter IV. Testes mass was the combined mean left and right testes masses with epididymis per species. The residual values for males were calculated by substituting data into the larger database in Dines et al. (2015). Measurements were collected from deceased adult specimens representing the same or nearby populations**.**

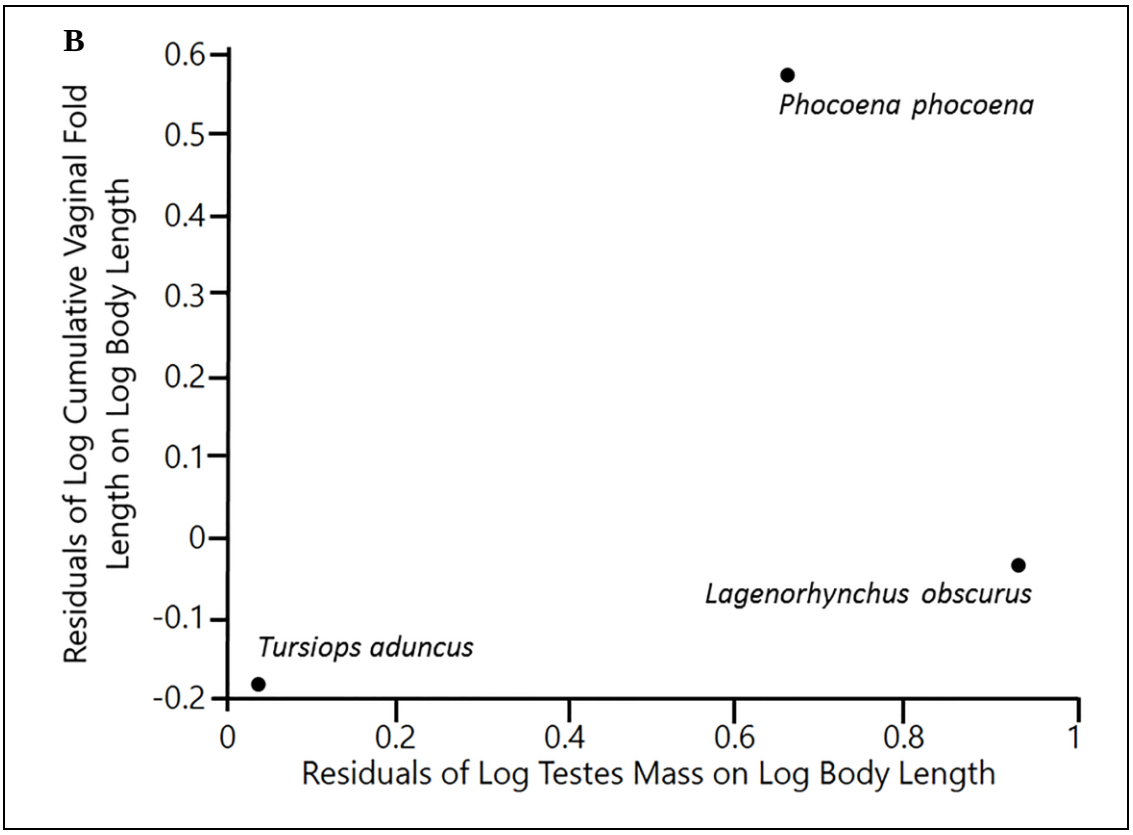

Figure 5-2, Continued.

Table 5-2. Patterns of mating group interactions, group size, and female behavioral effort in Indo-Pacific bottlenose dolphins, dusky dolphins, and harbor porpoises.

	Indo-Pacific Bottlenose Dolphin (*Tursiops aduncus*)	**Dusky Dolphin (*Lagenorhynchus obscurus*)**	**Harbor Porpoise (*Phocoena phocoena*)**
Mating Group Interaction Duration (seconds)	Mean ± S.D. = 91.6 ± 100.5	Mean ± S.D. = 624.8 ± 475.3	Mean ± S.D. = 7.3 ± 10.7
	Median = 47	Median = 503	Median = 4
	Range = 11 – 343	Range = 122 – 2467	Range = 2 – 52
Mean Copulation Rate (No. copulation attempts·min^{-1})	15.5	1.3	10.5
No. Males Group^{-1}	Mode = 2	Mode = 4	Mode = 1
	Range = 3 – 6	Range = 2 – 25	Range = 1 – 2
Female Behavioral Repertoire Size	6	6	6
Female Behavioral Intensity	Low	High	Low

of 92 sec. The mean copulation rate was 15.5 attempts·min^{-1}. The modal group size was 2 males and 1 female. Only 1 female was in each group, except for one occasion where 2 males and 2 females were present. Videos of 48 dusky dolphin mating groups yielded 29,191 sec of mating interactions with a mean duration of 625 sec. The mean copulation rate was 1.3 attempts·min^{-1}. The modal group size was 4 adult males and 1 adult female. On one occasion, a calf was present and on four occasions a sub-adult was present in the mating group. For harbor porpoises, 22 videos of mating groups yielded 160 sec of mating interactions with a mean duration of 7 sec. The mean copulation rate was 10.5 attempts·min^{-1}. The group size was consistently 1 adult male and 1 adult female, except for two occasions where a calf was present and one occasion where a second male was present.

Females in all three species had the same behavioral repertoire size consisting of 6 behavior types (Table 5-1). The behavior types were not the same across all three species (Table 5-1; Fig. 5-3). Female behavior types occurred at non-equal probabilities within Indo-Pacific bottlenose dolphins ($GF\text{-}X^2_5$= 428.11, $p < 0.001$; Fig. 5-3A), dusky dolphins ($GF\text{-}X^2_5$ = 965.75, $p < 0.001$; Fig. 5-3B), and harbor porpoises ($GF\text{-}X^2_5$ = 14.44, $p < 0.05$; Fig. 5-3C).

The variation in female behavioral intensity by species is shown in Figure 5-4. Given that a behavioral event was observed in a given species, it was not equally probable to be a high or low intensity event (G^2_2= 735.66, N = 1298, $p < 0.001$). Low intensity behaviors were associated with Indo-Pacific bottlenose dolphins (z = 20.79)

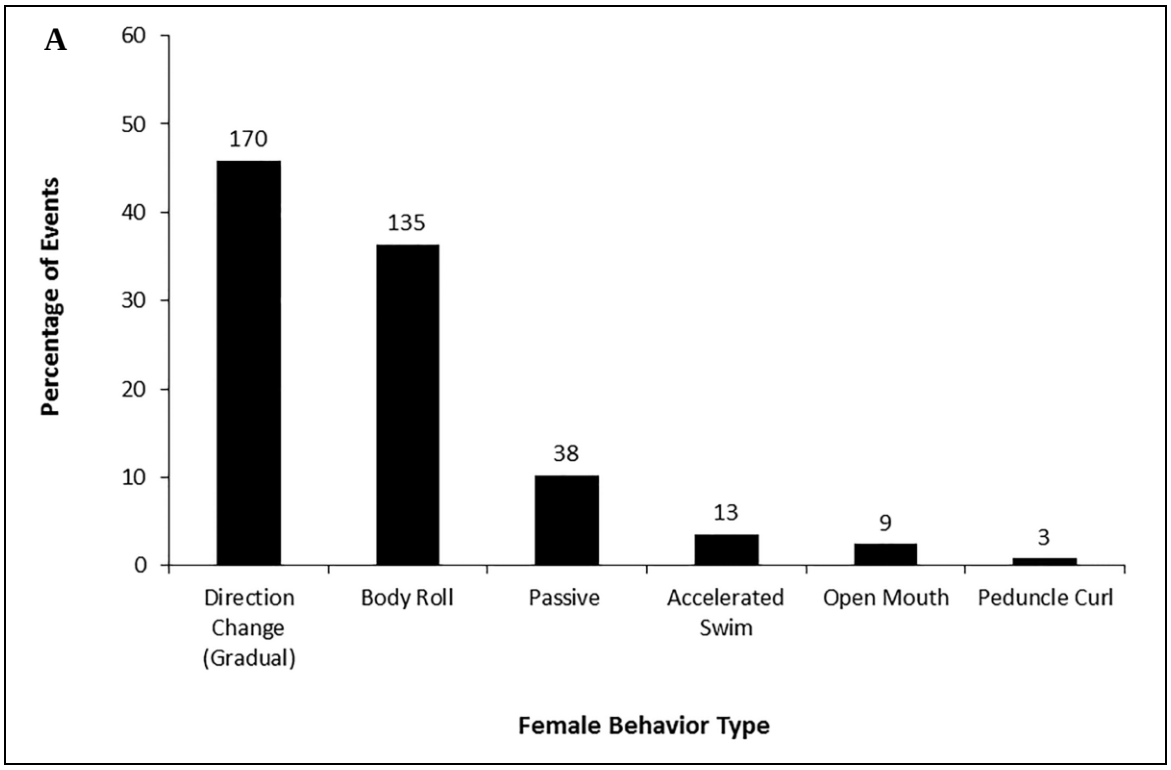

Figure 5-3. Frequency distributions of female mating behavior types for (A) Indo-Pacific bottlenose dolphins, (B) dusky dolphins, and (C) harbor porpoises. Frequencies are shown as percentages. The count of behavioral occurrences is listed above the bars for each behavior type.

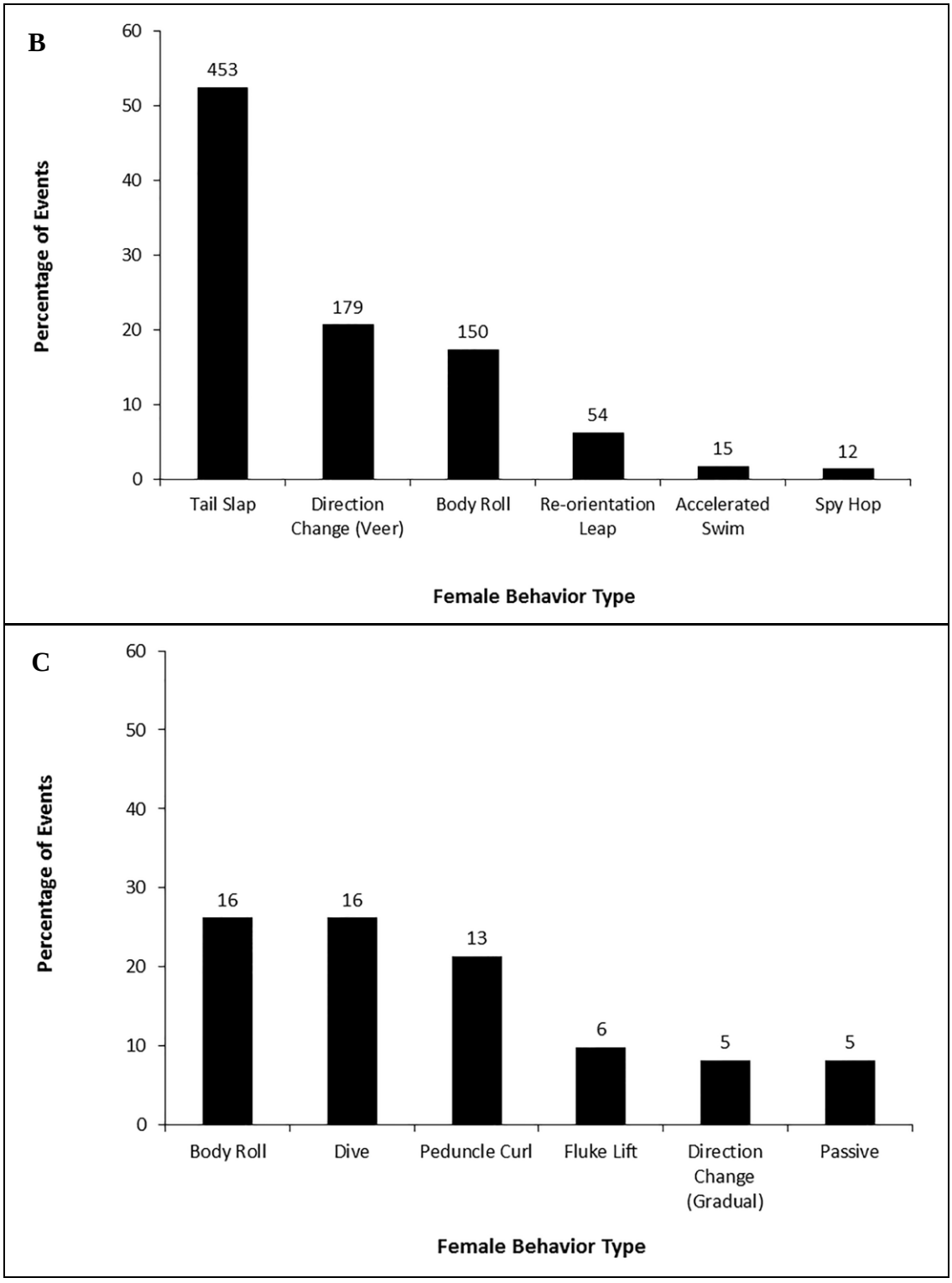

Figure 5-3, Continued.

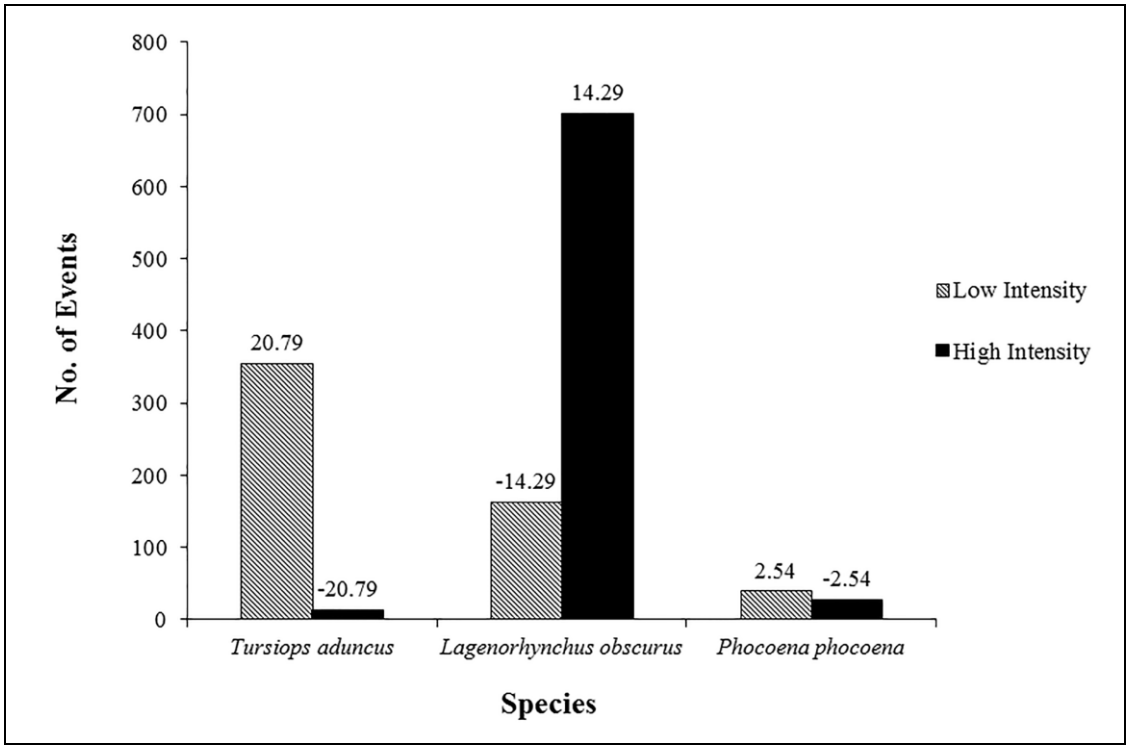

Figure 5-4. Counts of female behavior types per species by intensity. Behavior types are pooled into high or low intensity events. Intensity classifications were based on inter-rater reliability scores. Hashed bars denote low intensity behaviors and black bars denote high intensity behaviors. The binomial *z*-scores are included above the bars.

and harbor porpoises (z = 2.54), while high intensity behaviors were associated with dusky dolphins (z = 14.29; Fig. 5-4).

Discussion

A general trend was found between female and male reproductive anatomy using two measures of vaginal complexity across three species. Sexually mature common bottlenose dolphins (*T. truncatus*) had simple vaginal morphologies and small relative testes sizes, while dusky dolphins (*L. obscurus*) had intermediate vaginal complexity and large relative testes size, and harbor porpoises (*P. phocoena*) had relatively more complex vaginal morphologies and large relative testes sizes. As vaginal morphology varied in ways that could not be fully explained by phylogeny, body size, or physical environmental factors (chapter IV), and may relate to testes size, the vaginal folds of cetaceans may function in post-copulatory sexual selection. However, broader tests that correlate female and male reproductive morphology (including penis shape and size) across the phylogeny are needed to properly test for trait coevolution (e.g., Arnqvist and Rowe, 2002a; Brennan et al., 2007).

Female pre-copulatory traits did not follow the predicted pattern based on post-copulatory traits. There were no differences in behavioral repertoire sizes among female Indo-Pacific bottlenose dolphins (*T. aduncus*), dusky dolphins, or harbor porpoises, although there were differences in the frequency of occurrence of each behavior type within each species. Contrary to predictions, female dusky dolphins, not Indo-Pacific

bottlenose dolphins, exhibited the most frequent high intensity evasive behaviors in mating contexts. Behavioral predictions based on reproductive anatomy may not have been met because: 1) there are no trade-offs between pre- and post-copulatory traits for females, 2) different variables are necessary to evaluate variation in pre- and post-copulatory traits of females, or 3) socio-sexual and physical environmental factors have a strong influence on behavioral phenotypes.

Across many taxa where females mate with multiple males, trade-offs occur between resources allocated towards male traits that enhance acquisition of mates or fertilization success (e.g., Parker et al., 2013; flies, Markow, 2002; Puniamoorthy et al., 2012; crayfish, Galeotti et al., 2012; fish, Evans, 2010; bats, Pitnick et al., 2006; pinnipeds, Fitzpatrick et al., 2012; cetaceans, Dines et al., 2015; see Lüpold et al., 2014 for exceptions). The concept of limited energetic budgets for investment in reproduction has been applied differently to females, with an emphasis instead on the energetic demands of offspring production and care (Gittleman and Thompson, 1988; Emery Thompson, 2013) or costs and benefits of mate choosiness (Jennions and Petrie, 1997; Wong and Candolin, 2005; Cotton et al., 2006; Bleu et al., 2012). Spermatogenesis appears to be costly for cetaceans based on evidence of a seasonal regression in testes size for several species (Cipriano, 1992; Fontaine and Barrette, 1997; Neimanis et al., 2000; Murphy et al., 2005; Kelley et al., 2015), and disproportionate investments in traits that either enhance pre- or post-copulatory reproductive success (Connor et al., 2000a; Dines et al., 2015). The lack of variation in vaginal morphology between sexually immature and mature common bottlenose dolphins indicates that vaginal form

does not change (Orbach et al., 2016/chapter III) and vaginal tissue is not energetically costly to maintain. Thus, there may be no trade-off for females between reproductive anatomy and behavior, similar to the trend found in female waterfowl that have complex vaginal morphology and exhibit high energy pre-copulatory evasive behaviors (Brennan et al., 2010). Female cetaceans may use a range of pre- and post-copulatory mechanisms to control paternity within any given species.

Additional measures of female control over the outcome of mating encounters should be explored, as patterns could vary relative to predictions based on post-copulatory traits. For example, future comparative studies could assess the complexity of successive female behavioral events or how female behavioral efforts change with the repertoire size and intensity of male mating behaviors (e.g., Orbach et al., 2015a/chapter II). It would also be valuable to compare the amounts of time females spend resisting males per successful or unsuccessful copulation. In the present study, it was not possible to visually confirm if or when successful intromission and ejaculation occurred (e.g., southern right whales, *E. australis*; Würsig, 2000). The range and intensity of female behavioral effort is also expected to vary within species depending on the female's state of estrus, sexual maturity, and experience, which can subsequently alter her attractiveness and receptivity to males (Indo-Pacific bottlenose dolphins, Conner et al., 1996; humpback whales, *Megaptera novaeangliae*; Tyack and Whitehead, 1982; North Atlantic right whales, *Eubalaena glacialis*; Kraus and Hatch, 2001).

Lastly, variation in behavior within and across species may reflect physical (e.g., resource distribution) and socio-sexual (e.g., demographic) environmental factors. For

example, group size and composition can be an important determinant of mating patterns in cetaceans (e.g., Gowans et al., 2007). Female Indo-Pacific bottlenose dolphins exhibited more low than high intensity behaviors than predicted, including proceptive behaviors such as passive floating. Sexual size dimorphism is weak in this species (Wilson and Mittermeier, 2014), mating group sizes were small (mode = 2 males; Table 5-2), and males often alternated copulations with the female. If male mating tactics in the Hurghada population include alliance formation and sexual coercion, as found in some other populations of Indo-Pacific bottlenose dolphins, females may be relatively passive during mating interactions because of the potential to incur substantial direct costs to resisting copulations; these costs can include physical injury, harassment, lost foraging opportunities, and infanticide (Conner et al., 1992a, 1996; Connor and Smolker, 1996; Scott et al., 2005; Watson, 2005). Males appear to manipulate female acquiescence with threat vocalizations and displays (Connor and Smolker, 1996), and females may be constrained in their ability to exercise mate choice. The lack of evidence that female bottlenose dolphins invested heavily in pre- or post-copulatory traits may indicate that females have other means of controlling paternity, such as physiological mechanisms. For example, females might alter their number of estrous cycles and improve the probability of conception by preferred males in subsequent consortships (Connor et al., 1996).

Female dusky dolphins exhibited the most frequent high intensity evasive behaviors of the three focal species, although they were predicted to be intermediate based on vaginal complexity. The relatively large mating group sizes (mode = 4 males;

Table 5-2), low copulation rates (1.3 attempts·min^{-1}; Table 5-2), lack of sexual size dimorphism (Cipriano, 1992), low aggression levels towards females (Orbach et al., 2015b), lack of male cooperation (Orbach et al., 2015c), and spatially and temporally reliable prey patches that do not require resource defense (Dahood and Benoit-Bird, 2010) provide supporting evidence that female dusky dolphins have a low potential to be monopolized by males (Orbach et al., 2014). Since daytime foraging is rare off Kaikoura (Markowitz, 2004, 2012), females do not incur costs of lost foraging opportunities through time and energy allocated to evading males in mating chases. The direct costs of high intensity evasive behaviors appear to be lower for dusky dolphins than Indo-Pacific bottlenose dolphins, as predicted in Orbach et al. (2015a/chapter II).

Female harbor porpoises exhibited low behavioral intensity levels, as predicted by reproductive anatomy. The slightly reversed sexual size dimorphism (Read and Tolley, 1997), small mating group sizes (mode = 1 male; Table 5-2), brief mating group interactions (median = 4 secs; Table 5-2), and complex body alignment during copulation attempts (the male was partially aerial with his penis hooked under the ventrum of the female) may impede monopolization of females and are not indicative of coercion. Additionally, the low intensity of female behaviors may reflect data collection outside the peak breeding season, during which costs to females are expected to be comparatively low. These factors may contribute to more frequent low intensity behaviors than expected by chance among female Indo-Pacific bottlenose dolphins and harbor porpoises.

Features of the study design may also account for some of the variation in patterns between pre- and post-copulatory traits. For example: 1) pre- and post-copulatory traits could not be assessed in the same species of bottlenose dolphins due to logistical challenges of obtaining reproductive tracts of *T. aduncus* specimens or observing the mating behaviors of *T. truncatus* populations (e.g., rare beach-cast stranding events, permits, poor water visibility, etc.). However, vaginal morphology is relatively conserved within a genus (appendix A); 2) females were assumed to be similar in estrus state, age class, and experience across focal follows, which could influence attractiveness to males and behavioral repertoires; 3) mating events were assumed to be procreative in nature, although socio-sexual behaviors can occur year-round in cetaceans (including outside the breeding season) and serve many functions (e.g., conception, teaching, play, establishing social bonds; Mann, 2006); 4) the impacts of the research vessel and underwater divers on mating behaviors could not be directly determined. However, mating groups appeared to be unperturbed and continued chasing each other and attempting copulations. Non-invasive viewing platforms, such as bridges and unmanned aerial vehicles, can help mitigate possible observer effects; 5) different viewing platforms for the three species could compromise visibility of certain behavior types. However, dusky dolphins in mating groups off Kaikoura spent 91% of the time at the surface of the water, where they were visible (Orbach et al., 2015a/chapter II). Male harbor porpoises appeared to approach and depart from females immediately prior to and after copulation attempts, with no additional interactions with females. Additionally, copulation attempts consistently coincided with the female's surfacing to breathe,

suggesting the water's surface was an important factor for harbor porpoise mating attempts. Future studies that can control for some of these variables and assess additional species (and several populations within one species) are needed.

Studies of the relationship between pre- and post-copulatory trade-offs within one sex have opened the possibility to assess trade-offs in the other sex and explore the broader patterns of mating strategy coevolution. While this study focused on post-copulatory traits of both sexes and pre-copulatory traits of females, an integrative approach that includes the pre-copulatory traits of both sexes is recommended for future research. The comparative approach is a valuable tool to highlight the role of sexual selection in driving genital evolution and covariance with pre-copulatory traits. The patterns of pre- and post-copulatory traits found in bottlenose dolphins, dusky dolphins, and harbor porpoises may subsequently be applied to predict the mating tactics of other cetacean species. However, environmental factors should be considered due to the high degree of behavioral variability in dolphins and porpoises.

CHAPTER VI

CONCLUSIONS

This dissertation adds to the body of knowledge of cetacean sociobiology through development and empirical assessment of female mating strategies from proximate and ultimate perspectives, and through evaluation of the coevolution of anatomy and behavior, both within and between the sexes. The correlated evolution of male and female mating strategies has been studied in a broad range of taxa (e.g., Wiley and Poston, 1996; insects, Arnqvist and Rowe, 2002a; fish, Houde and Endler, 1990; waterfowl, Brennan et al., 2007; primates, Nunn, 1999; ungulates, Bro-Jørgensen, 2011). While logistical constraints of data collection make cetaceans particularly challenging to study (Lanyon and Burgess, 2014), they provide a rich clade within which to examine patterns and processes of evolution in aquatically mating mammals.

This dissertation addresses mating behavior, reproductive anatomy, and trade-offs between the two. In chapter I, I proposed a heuristic framework for the coevolution of male and female mating strategies in cetaceans and used dusky dolphins (*Lagenorhynchus obscurus*) as a model species. Specifically, I proposed that males have low monopolization potentials of females, females evolved evasive behavioral maneuvers, males evolved large relative testes sizes, and females evolved convoluted vaginas. This dissertation focused on the female components of the framework, as the

male components have been previously validated for dusky dolphins (Cipriano, 1992; Van Waerebeek and Read, 1994; Orbach et al., 2014, 2015c).

Chapter II showed that female dusky dolphins demonstrated frequent active evasive behaviors in the context of exploitative scramble competition, which suggests low female receptivity for males. Females did not exhibit any affiliative behaviors (e.g., rubbing) and the only non-evasive behavior displayed was copulation, which might not be consensual for the female. Females were resistant to copulations in most (83%) sequences with copulatory body positioning. The structures of behavioral sequences were more complex when females terminated the copulation attempt compared to when females did not terminate the attempt. Additionally, females displayed non-random responses to male signals throughout mating group follows. Collectively, the data indicate that females evolved evasive behavioral maneuvers.

Chapter III developed a protocol to dissect and collect 15 standardized measurements of female cetacean reproductive tracts. The reproductive tracts of common bottlenose dolphins (*Tursiops truncatus*, N =18) were assessed instead of dusky dolphins because of a large sample size availability. Vaginal morphology was similar across sexual maturity and reproductive states within one species. The microstructure of vaginal folds revealed smooth and not skeletal muscle bands, indicating autonomic control of muscular contractions. However, the lack of skeletal muscle does not preclude the possibility that females can selectively move semen within their vaginas by peristaltic smooth muscle contraction. Chapter IV assessed scaling relationships and the diversity of vaginal morphology across 19 species of cetaceans while controlling for

phylogenetic relatedness. Dusky dolphins had intermediate vaginal lengths and cumulative vaginal fold lengths compared to other species, and were close to or on the regression line. Both vaginal length and cumulative vaginal fold length were positively related to body length, but not to each other. Selective forces appear to act differently on these two vaginal traits. The data from chapters III and IV validate that cetaceans have convoluted vaginas (compared to other mammals), and provide techniques to measure and compare vaginal complexity. These two chapters lay the foundation to evaluate the function(s) of vaginal folds, including a role in sexual selection.

Chapter V used a comparative approach to investigate the association between reproductive anatomy and behavioral traits in three species. A species with small testes had simple vaginal morphology (*T. truncatus*) and two species with large testes had relatively more complex vaginal morphologies (*L. obscurus, Phocoena phocoena*). However, female pre-copulatory traits (mating behavior repertoire size and intensity) did not match the trends predicted based on post-copulatory traits. Female dusky dolphins demonstrated more behavioral mating effort than harbor porpoises or Indo-Pacific bottlenose dolphins (*T. aduncus*). Differences in female behaviors across species may be best explained by varying environmental conditions. Females appear to use a variety of pre- and post-copulatory mechanisms to control paternity.

Overall, the data presented in this dissertation provide support for the proposed heuristic framework for the coevolution of male and female mating strategies in dusky dolphins; males had a low potential to monopolize females and large relative testes size, while females were evasive and had relatively complex vaginas. Bottlenose dolphins

demonstrated opposite patterns; males had a higher potential to monopolize females and relatively small testes size, while females were comparatively non-evasive and had simple vaginal morphologies. Harbor porpoises provide yet another combination of pre- and post-copulatory traits; males had a relatively low potential to monopolize females yet rather large testes size, and females were comparatively non-evasive yet had complex vaginal morphologies. These three examples highlight the species- and possibly population-specific dynamics of mating tactics among cetaceans. The physical environment and particularly the socio-sexual competition environment appear to play large roles in shaping the evolution of cetacean mating tactics. For example, a male's monopolization potential of rivals' access to females may be predicted from local ecological conditions that influence the ranging patterns and the stability of female group sizes (Gowans et al., 2007). Additional populations and species should be examined to further identify potential causes of diversity in mating strategies and to build a predictive model widely applicable to cetaceans.

Importance of Research

This dissertation provides broad insights on aspects of sexual selection in cetaceans, with practical applications to other taxonomic groups. I provide a mechanism to assess the potential for active female mate choice by evaluating how females respond behaviorally to male signals (Markov chain analyses, chapter II). My research addresses the sex bias in studies of genital evolution. I demonstrate that female genitalia in cetaceans are more complex than commonly recognized (reviewed in Ah-King et al., 2014), warrant further

investigation across taxa, and can potentially function in post-copulatory sexual selection (chapters III and IV). Through characterization of the vaginal fold structure, I provide an innovative approach to assess the potential role of vaginal structures in cryptic female choice (histological analysis, chapter III). My pilot study also lays the foundation for computed tomography (CT) scans to evaluate vaginal morphology in 3-dimension (chapter III supplement). Chapter IV is one of the few studies to evaluate female reproductive tract scaling in any vertebrate species. Additionally, the data presented are the most comprehensive compilation of vaginal fold diversity within cetaceans. I also demonstrate how form can be used to derive an enhanced grasp of functionality in this context. I provide an approach to develop and empirically explore predictions for the coevolution of anatomy between the sexes, and of anatomy and behavior within one sex (chapter V). Genitalia can provide important insights into cetacean mating strategies, mating systems, and evolution- particularly in species that are cryptic or are not easily observed mating.

Recommendations for Future Research

Several remaining gaps in our knowledge of behavior and anatomy have the potential to broadly impact theoretical and empirical frameworks, and present exciting challenges for future studies of cetacean mating strategies. I add the caveat that this list is not exhaustive and that logistical challenges may be a hindrance to advancements (Lanyon and Burgess, 2014).

Behavior

Additional explorations of the mating behaviors of free-ranging cetaceans are needed to better grasp how animals adapt to physical and socio-sexual competition environments. The extensive diversity in the behavior repertoire types of female Indo-Pacific bottlenose dolphins, harbor porpoises, and dusky dolphins (chapter V) reinforce that environmental factors play a large role in shaping population-specific mating tactics. There are likely many additional mating tactics used by male and female cetaceans beyond those listed in Tables 1-1 and 1-2. Many reports of cetacean sexual activity are anecdotal or based on animals in captivity (e.g., Payne, 1995; Hills et al., 2015). Some proposed mating tactics are also debated (e.g., the function of narwhal tusks, *Monodon Monoceros*; Best, 1981; Gerson and Hickie, 1985). The challenges of observing cetacean copulation events in the wild can be extensive. Marine mammalogists have spent decades studying cetaceans during peak breeding seasons with rare observations of intromission events. Poor water visibility can be a major hindrance. Additionally, cetaceans may copulate while submerged and out of view from observers at the surface (e.g., Indo-Pacific bottlenose dolphins, chapter V). Recent advances in animal-borne video-equipment (e.g., Marshall et al., 2007) could revolutionize methods to acquire videos of cetacean copulation events. Underwater-based behavioral surveys during scuba dives may also be a useful method for future data collection (e.g., chapter V). However, non-invasive observational platforms (e.g., the Golden Gate Bridge, chapter V) are ideal to mitigate observer effects. Unmanned aerial systems have recently become more accessible for marine

mammal research and may be a valuable method for surface focal follows if implemented responsibly (Smith et al., 2016).

The ability to continuously track a focal female throughout her estrous cycle and document all copulation events, combined with extensive paternity testing, will be valuable additions to understanding the evolution of female mating strategies. I demonstrated that female dusky dolphins can discriminate between male behaviors (Orbach et al., 2015a/chapter II). However, data on male identity, individual variation in male mating behaviors, male variation in traits, and differential response of females to individual males are needed to demonstrate female mate choice. Studies that link observed mating patterns to reproductive success (proximate) and male seasonal mating success to individual variation in the lifetime reproductive success (ultimate) are also necessary. Knowledge of which copulation sequences lead to conceptions will likewise improve the comprehension of possible female criteria for mate selection. Cetaceans are not conducive to experimental manipulations, where male morphological traits can be physically altered and female preferences can be monitored (e.g., green swordtail fish, *Xiphophorus hellerii*; Basolo, 1990; barn swallows, *Hirundo rustica*; Møller, 1990).

There is still much work to be done to distinguish if some female mating behaviors function to reduce harassment from males or to screen the competitive abilities of prospective mates, although they are not necessarily mutually-exclusive. For example, the high speed mating chases led by female dusky dolphins (Orbach et al., 2015a/chapter II) appear to be avoidance behaviors, but could also function as a mechanism for females to 'evaluate' male endurance and vigor (Whitehead and Mann,

2000). In North Atlantic right whales (*Eubalaena glacialis*), the vocalizations produced by females in surface active groups have been hypothesized to incite males to join the mating competition (Kraus and Hatch, 2001); females can then select for male stamina and ability to displace rivals, as observed in northern elephant seals (*Mirounga angustirostris*; Cox and Le Boeuf, 1977). The ability to disentangle intersexual conflict from cooperation is a complex topic in sexual selection theory that requires further exploration.

A major knowledge gap is how to behaviorally determine a female's state of estrus, which peaks at the time of ovulation and alters her receptivity to males (Petrulis, 2013). In addition to condition-dependent factors (e.g., genetic quality, nutrition state, age), a female's estrus state can have a large influence on mate preference, behavioral mating effort, and the coevolutionary dynamic of male and female mating strategies (Cotton et al., 2006). Female reproductive status is a strong determinant of occurrence in mating groups (e.g., North Atlantic right whales, *Eubalaena glacialis*; Kraus and Hatch, 2001). Ovulatory state has been determined hormonally in some cetaceans (Robeck et al., 1994; Rolland et al., 2005; Steinman et al., 2012), although phenotypic indicators have not been confirmed (reviewed in Orbach et al., 2014). It is also unclear which signals trigger ovulation and if signals vary across species. Ovulations can be spontaneous (e.g., false killer whales, *Pseudorca crassidens*; Atkinson et al., 1999; common bottlenose dolphins, *T. truncatus*; Kirby and Ridgway, 1984; Muraco et al., 2010; common dolphins, *Delphinus delphis*; Kirby and Ridgway, 1984*:* killer whales, *Orcinus orca*; Robeck et al., 1993) or induced (e.g., beluga whales, *Delphinapterus*

leucas; Steinman et al., 2012). It is not known if the presence of a male is sufficient to induce ovulation or if he supplies specific cue(s) above a stimulation threshold (e.g., scent urination only by large males in moose, *Alces alces*; Miquelle, 1991).

Advanced techniques may be needed to confirm when ejaculation occurs during copulation attempts. While penis muscle contractions have been observed during cetacean copulations (e.g., Southern right whales, *E. australis*; Würsig, 2000), I could not confidently distinguish successful from unsuccessful copulation events during video analyses; the penis could not be visualized once it penetrated the vagina or when dusky dolphins swam ventrum-to-ventrum. Copulation durations were only a few seconds, were briefer in cetaceans than other mammals, and scaled negatively with body size (Stallmann and Harcourt, 2006). Subsequently, copulation duration may not be a reliable indicator of ejaculation and semen transfer.

Anatomy

The diversity of vaginal fold morphology requires further exploration in cetaceans. While chapter IV presents data on 19 of 90 extant cetacean species and is the most comprehensive review to date, additional data, including specimens obtained from different populations of the same species, may yield increased statistical resolution and new patterns. Vaginal folds were present in all specimens dissected during this study and in all reviewed publications. However, the possibility exists that vaginal folds may be absent in species that have not yet been examined. New techniques can also be implemented to explore vaginal fold form and provide perspectives unavailable when

reproductive tracts are dissected (e.g., the angle of projection of the vaginal fold into the lumen). Diagnostic imaging of reproductive tracts is a quickly advancing area of research that has been applied to cetaceans (e.g., Robeck et al., 1994, 2010; Brook, 1997; reviewed in Lanyon and Burgess, 2014). Techniques, such as CT scans, can provide 3-dimensional images of vaginal folds *in situ* (chapter III supplement), and may help to elucidate functionality.

This dissertation lays the foundation to explore possible functions of vaginal folds. Several techniques can now be implemented to test the hypothesis that vaginal folds play an important role in post-copulatory sexual selection, in addition to alternative functions. For example, a large-scale phylogenetic comparison of vaginal complexity and relative testes size could demonstrate if male and female reproductive morphology are correlated and under similar selection pressures. Similarly, the relationship between reproductive tract morphology and pre-copulatory mating behaviors (chapter V; Connor et al., 2000a) should be expanded to include additional species. I selected the number of vaginal folds and cumulative vaginal fold length as measures of vaginal complexity (chapter V), but other features of vaginal folds may improve the understanding of functions, including the shape, thickness, and positioning of vaginal folds relative to other vaginal landmarks.

Further research is needed to disentangle the possible functions of vaginal folds in controlling semen and water movement through the vagina. Physical vaginal models (i.e., casts) can be designed to assess where injected seawater and live semen pool respectively. Computed tomography techniques may be applied to create 3-dimensional

scans and printed model cetacean vaginas for future tests of functionality. Similarly, *in vivo* vaginal endoscopy of recently mated cetaceans can illuminate if and where vaginal folds trap semen and water. Semen can also be tagged and tracked through the female reproductive tract *in vivo* (e.g., fruit flies, *Drosophila melanogaster*; Manier et al., 2010) to better recognize its interactions with vaginal structures.

Additional investigations of the microstructure of vaginal folds are warranted, including assessment of the muscle architecture, presence and distribution of secretory and non-secretory tissues, and innervation. While I did not find evidence that vaginal fold muscles are of somatic origin (chapter III), the presence of smooth muscles suggest that peristaltic contractions may occur, and could move semen. If muscle contractions of vaginal folds are under autonomic control, females may not discriminate among the sperm of different mates, in support of a sexually antagonistic coevolution framework (as opposed to cryptic female choice). .

Finally, more data are needed on penile morphology in cetaceans. Males possess a fibroelastic penis, a characteristic shared with their closest relatives, even-toed ungulates. No studies have assessed copulatory fit (how deep the penis penetrates the vagina and which anatomical landmarks are in contact and where) or how morphological shapes influence copulatory interactions in cetaceans. Penis length may be an important factor to consider in explorations of the evolution and diversity of vaginal folds. For example, male dusky dolphins copulate with females in a ventrum-ventrum body alignment, Indo-Pacific bottlenose dolphins mount females on their side in a t-shaped formation, and harbor porpoises mount females from behind and hook their penises

around the female's body (chapter V). I hypothesize that penis morphology coevolves with copulatory positioning in cetaceans. I emphasize the need to integrate anatomical and behavioral considerations in the exploration of evolutionary patterns of mating strategies.

To conclude, the evolution of mating strategies is a rich area of research with multifarious opportunities for inter-disciplinary approaches, theoretical and empirical hypothesis testing, and many unresolved questions with the potential to revolutionize the field. We are at the cusp of a more profound understanding of the relationships of male and female mating strategies than we could have imagined even ten years ago. The key to a deeper comprehension of mating strategies will benefit from an integrative approach that relates diverse aspects of cetacean biology, including behavior, anatomy, physiology, genetics, and ecology, within an evolutionary context.

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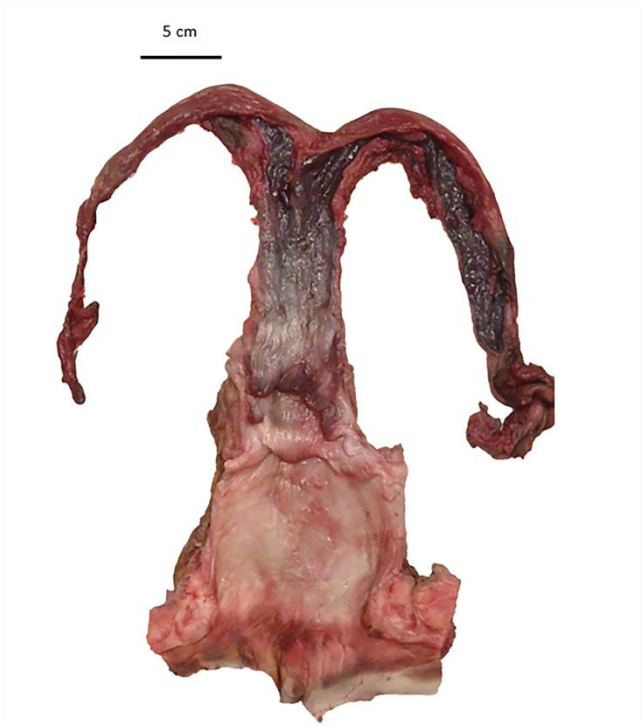
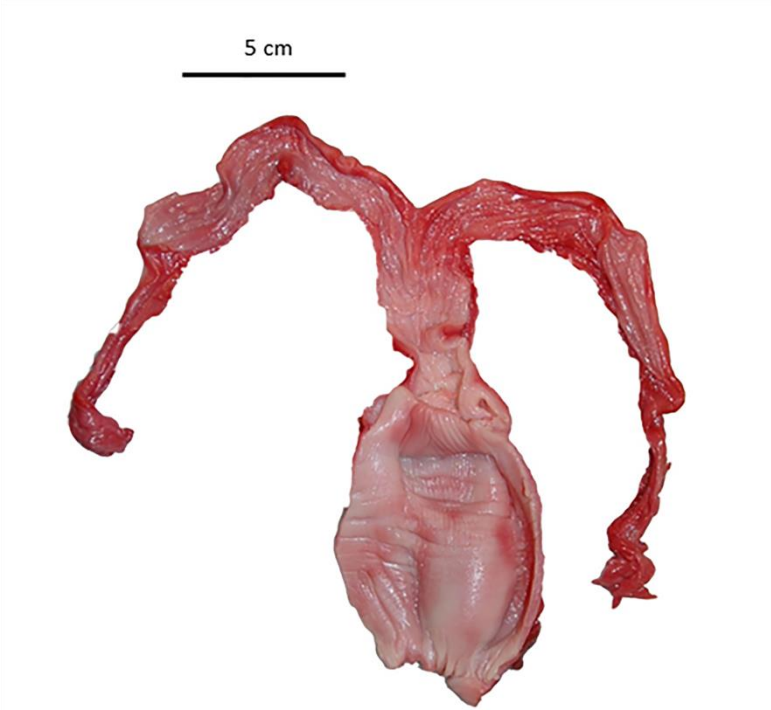
APPENDIX A

A-1. Figures of female reproductive tract diversity across 19 species of cetaceans.



Common bottlenose dolphin, *Tursiops truncatus*

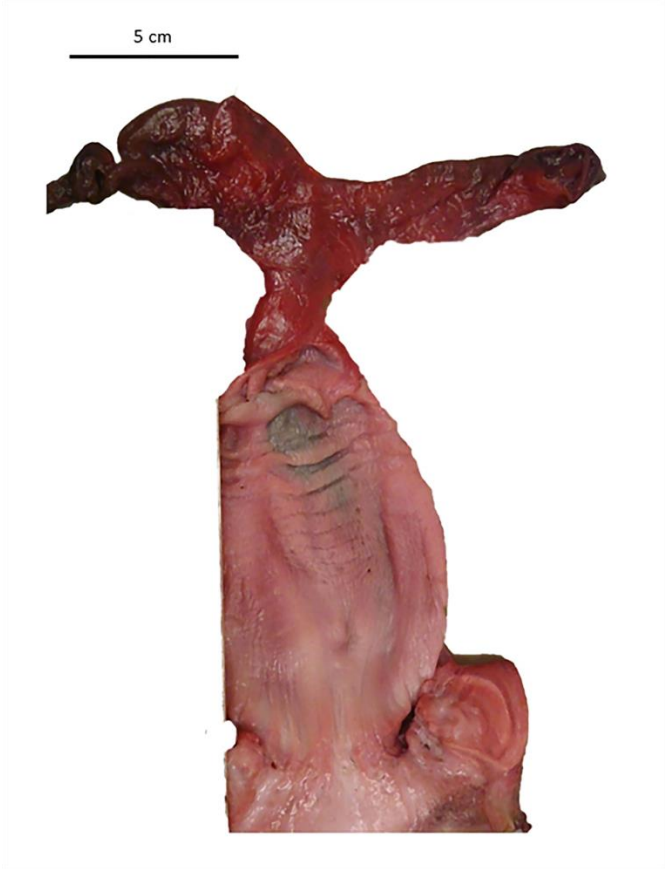


Atlantic spotted dolphin (Stenella frontalis)[3]

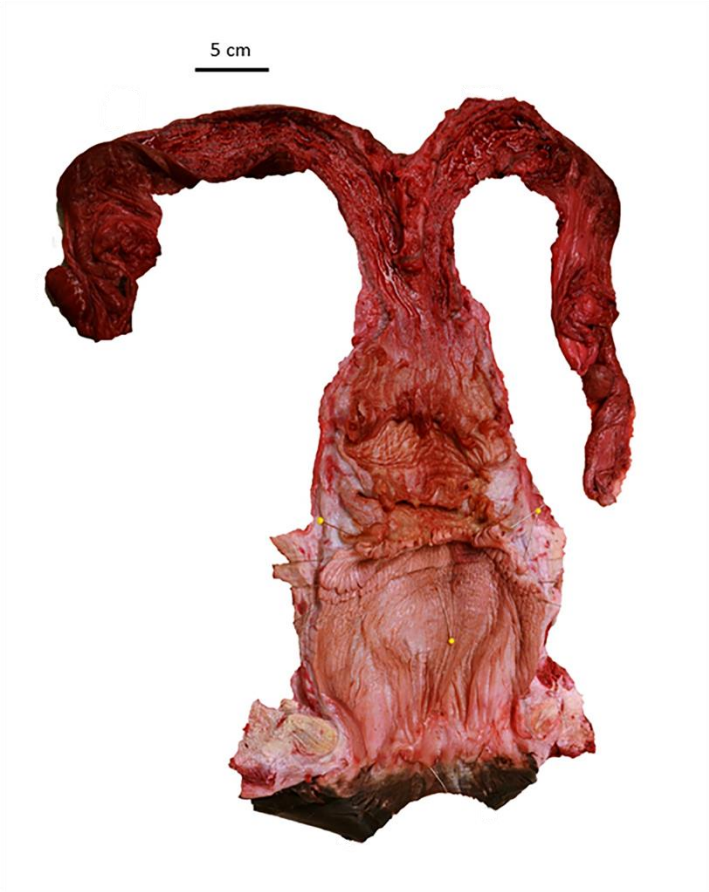
[3] Sub-adult specimen

Short-beaked common dolphin (*Delphinus delphis*)

Long-beaked common dolphin (*Delphinus capensus*)

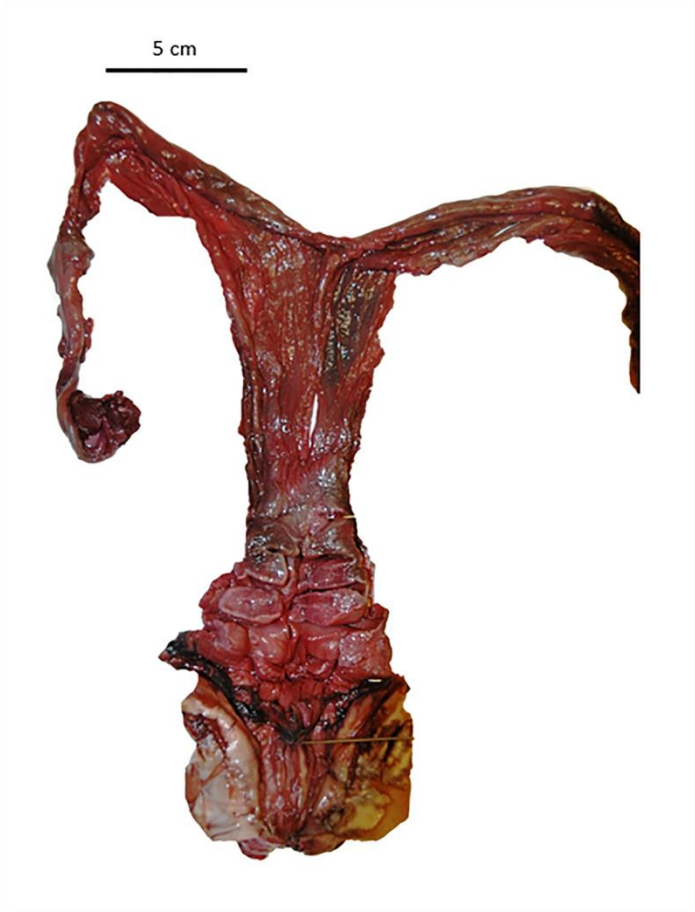


Long-finned pilot whale (*Globicephala melas*)[4]

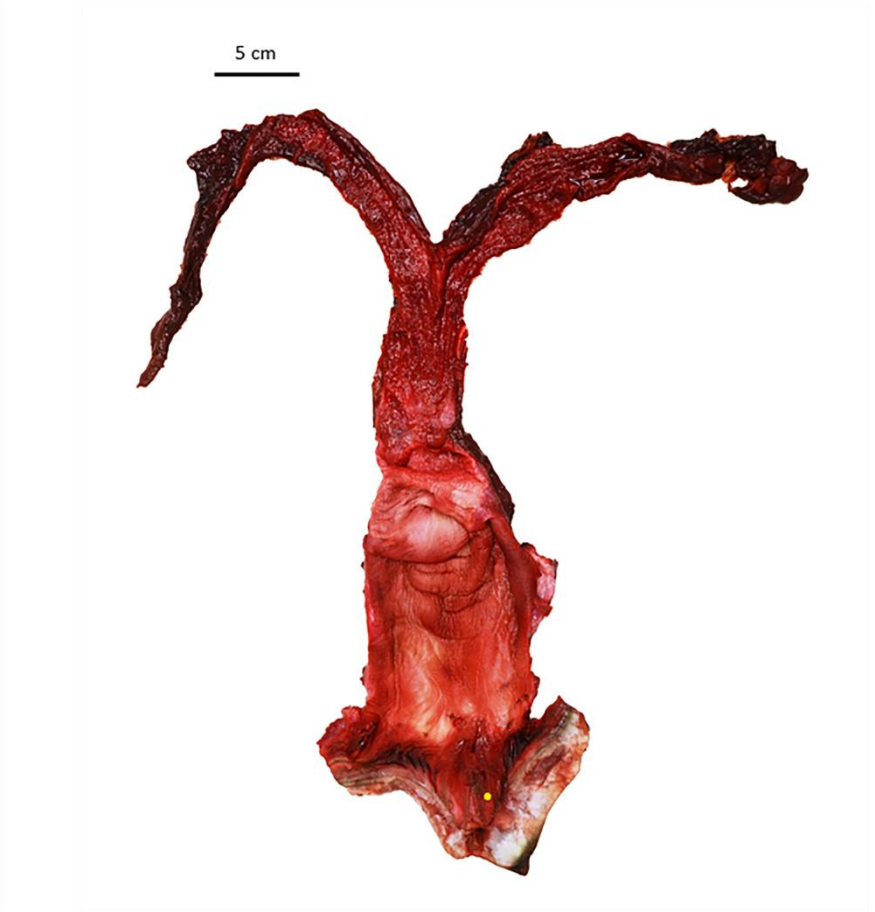


Short-finned pilot whale (*Globicephala macrorhynchus*)

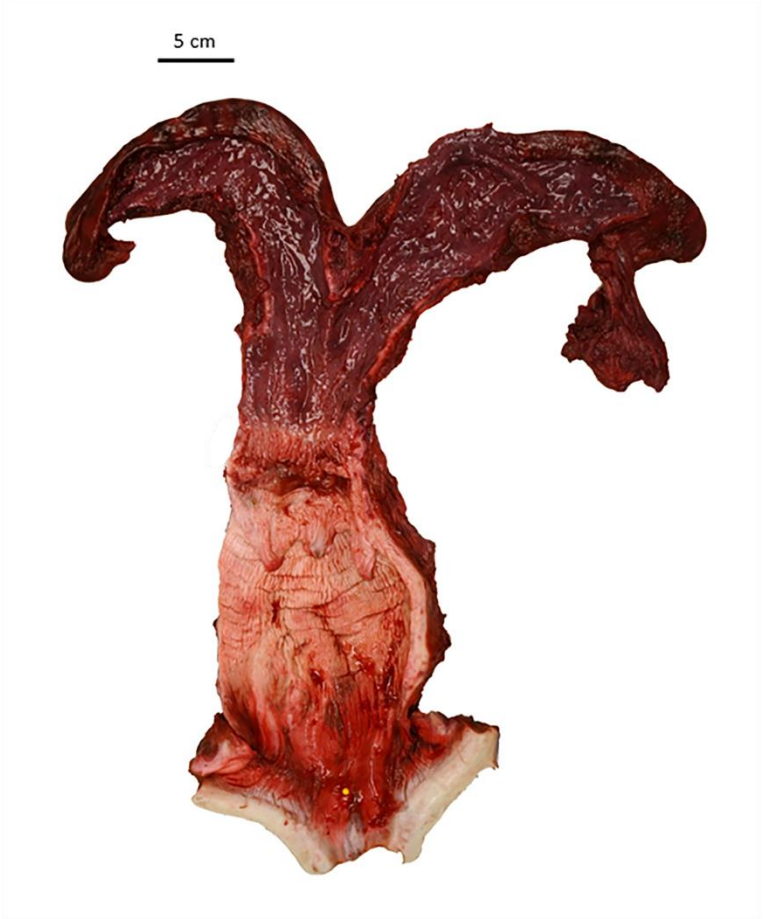
[4] Sub-adult specimen



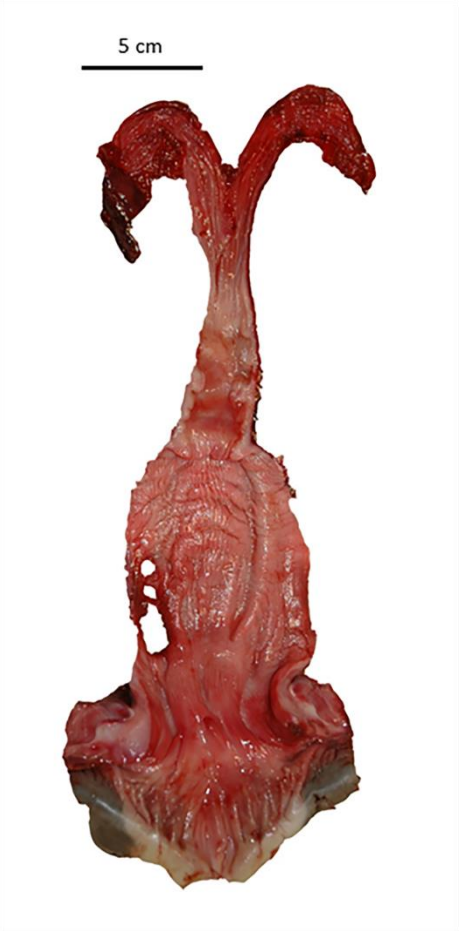
Dusky dolphin (*Lagenorhynchus obscurus*)



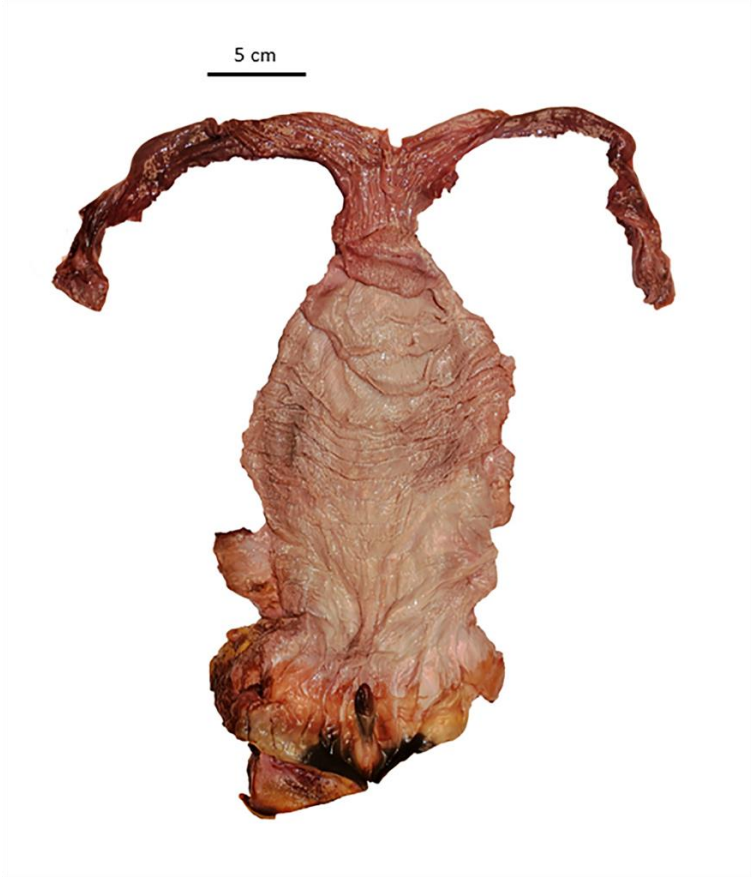
Pacific white-sided dolphin (*Lagenorhynchus obliquidens*)



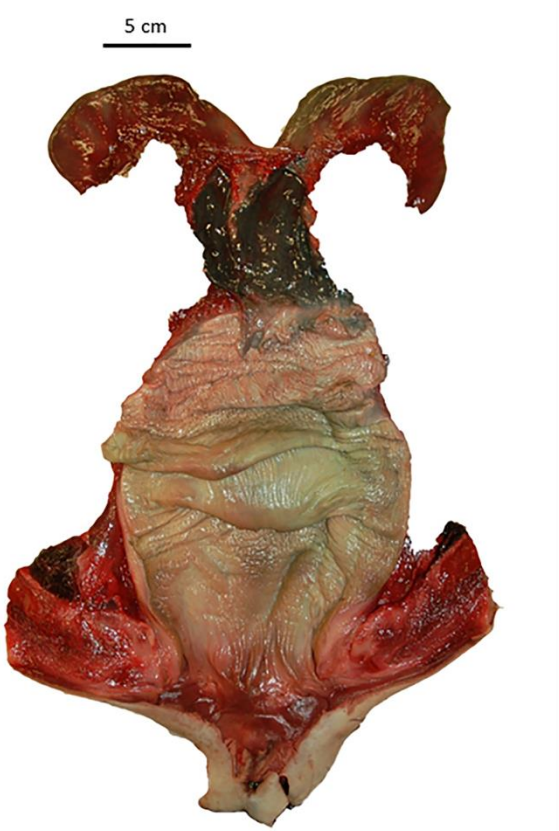
White-beaked dolphin (*Lagenorhynchus albirostris*)



Atlantic white-sided dolphin (*Lagenorhynchus acutus*)

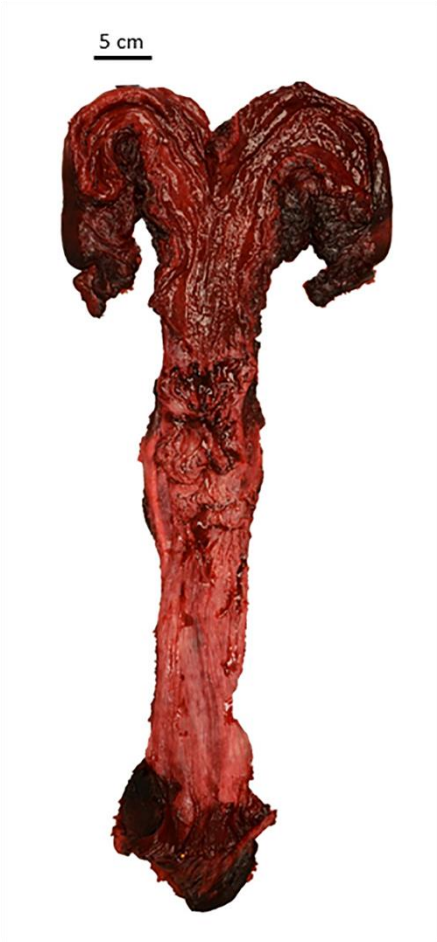


Killer whale (*Orcinus orca*)[5]



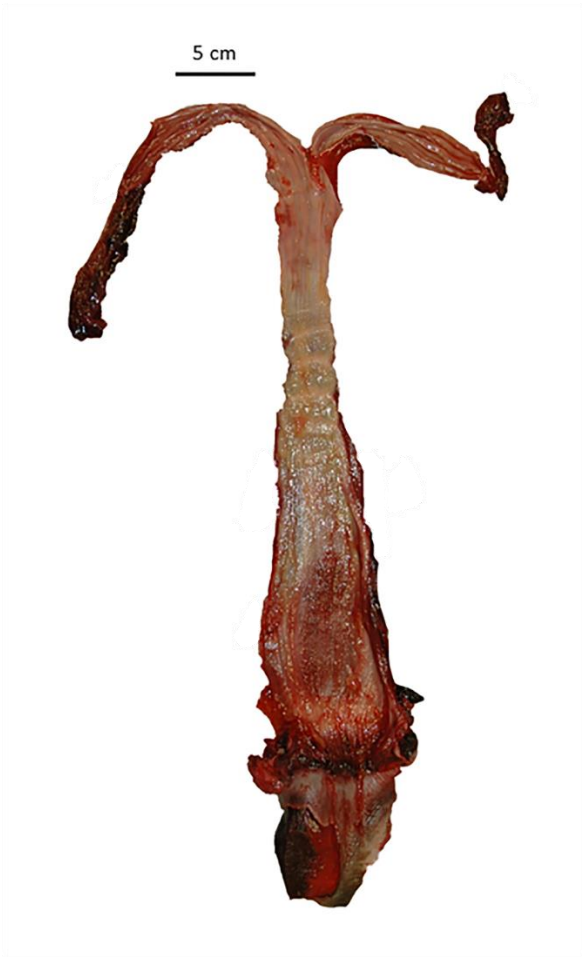
Harbor porpoise (*Phocoena phocoena*)

[5] Sub-adult specimen



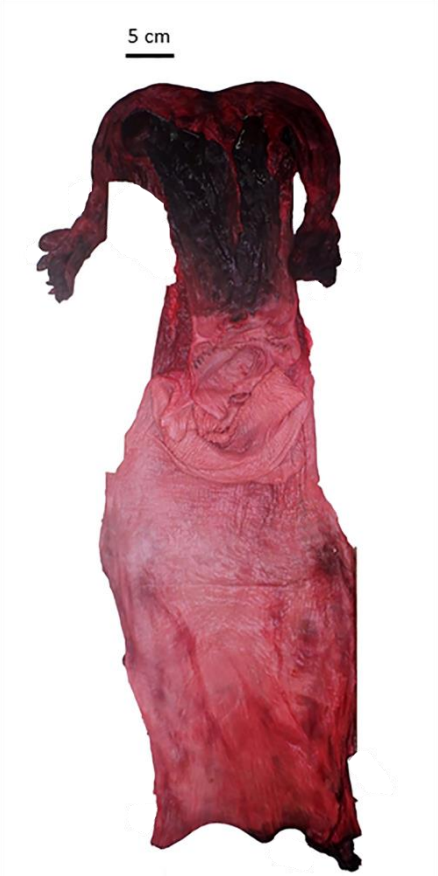
Stejneger's beaked whale (*Mesoplodon stejnegeri*)

Pygmy beaked whale (*Mesoplodon peruvianus*)



Gervais' beaked whale (*Mesoplodon europaeus*)

Sowerby's beaked whale (*Mesoplodon bidens*)



Pygmy sperm whale (*Kogia breviceps*)

Dwarf sperm whale (*Kogia sima*)[6]

[6] Sub-adult specimen

Common minke whale (*Balaenoptera acutorostrata*)